UNDERSTANDING THE ROLE OF SCL IN EARLY MAMMALIAN DEVELOPMENT USING MOUSE EMBRYONIC STEM CELL DIFFERENTIATION AS A MODEL

Approved by Supervisory Committee

Michael Kyba, Ph.D.

James Amatruda, Ph.D.

Michelle Tallquist, Ph.D.

Ondine Cleaver, Ph.D.

DEDICATION

This work is dedicated to my family.

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by

Ismail Ismailoglu

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Ismail Ismailoglu Ph.D

The University of Texas Southwestern Medical Center at Dallas, 2010

Michael Kyba Ph.D.

How a complete organism develops from a single cell is among the most complicated questions in life sciences. Early experimental studies on the development of animals were performed on amphibians and birds due to the size and accessibility of their embryos, while studies in placental mammals have been limited by the difficulty posed by in utero development.

In vitro differentiation of ES cells provides a convenient model for the study of the mammalian development. Since ES cells can be grown and maintained in a pluripotent state virtually forever, ample amount of research material for molecular

biological studies can be produced; differentiating ES cells are easily accessible and they can also be manipulated genetically.

I have used the ES cell differentiation model to study the bHLH factor SCL, a critical regulator of the formation of the hematopoietic lineage in the early embryo and the maturation of erythrocytes and megakaryocytes later on. The latter function of the protein has been studied extensively, but a complete molecular analysis of the former function has been lacking.

My work shows that SCL can skew the patterning of the mesoderm towards the hematopoietic lineage. This function required the interaction of SCL with LMO2. Transcriptional profiling revealed organizer genes FoxA2 and Chordin as novel downregulated targets of SCL during this time.

Differentiation of human pluripotent cells to be used in cellular therapy or to generate replacement tissues; is considered to be one of the most promising branches of medical research.

Considering the importance of SCL in hematopoiesis, we hypothesized that SCL can direct differentiation of pluripotent cells to this lineage in a simple culture system. Ectopic expression of SCL induced hematopoiesis at low levels. Co-expression of LMO2 and GATA2 increased efficiency of the programming significantly.

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PRIOR PUBLICATIONS

1. Ismailoglu I, Yeamans G, Daley GQ, Perlingeiro RC, Kyba M. Mesodermal patterning activity of SCL. Exp Hematol. 2008;36:1593-1603.

2. Ismailoglu I, Kyba M. Highly efficient programming of embryonic stem cells to blood by a transcription factor cocktail. *Submitted*

3. Ismailoglu I, Kyba M. SCL interacts with LMO2 in early hematopoietic development to repress the expression of node genes FoxA2 and Chordin. *In preparation*

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LIST OF DEFINITIONS

AGM – Aorta-Gonads-Mesonephros

AVE – Anterior visceral endoderm

bHLH – basic Helix Loop Helix

BL-CFC – Blast Colony Forming Cell

BMP – Bone Morphogenetic Protein

ChIP – Chromation Immunoprecipitation

CFC – Colony Forming Cell

DLP – Dorsal Lateral Plate

DMEM – Dulbecco's Modified Eagle's Medium

Dox - Doxycycline

EB – Embryoid body

EryP – Primitive Erythrocyte

ES cells – Embryonic stem cells

ExE – Extraembryonic Ectoderm

FACS – Fluorescent Activated Cell Sorting

FBS – Fetal Bovine Serum

FGF – Fibroblast Growth Factor

Flk – Fetal Liver Kinase

GFP – Green Fluorescent Protein

HDAC – Histone Deacetylase

HRP – Horseradish Peroxidase

HSC – Hematopoietic Stem Cell

ICM – Intracellular Mass

Id – Inhibitor of differentiation

Ihh – Indian Hedgehog

IMDM - Iscove's Modified Dulbecco's Medium

IP - Immunoprecipitation

iPS cells – induced Pluripotent Stem cells

iSCL – inducible SCL

KOSR – Knockout Serum Replacement

LIF – Leukemia Inhibitory Factor

LTR-HSC – Long Term Repopulating-Hematopoietic Stem Cell

MEL – Murine Erythroleukemia

MEF – Mouse Embryonic Fibroblast

MTG - Monothioglycerol

NEAA – Non-Essential Amino Acids

PAGE – Polyacrylamide Gel Electrophoresis

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDGFRα – Platelet Derived Growth Factor Receptor α

PI – Propidium Iodide

P/S – Penicillin/Streptomycin

qRT-PCR – quantitative Real-Time Polymerase Chain Reaction

RIPA – Radioactive Immunoprecipitation Assay

RT – Room Temperature

SCL – Stem cell leukemia

SDS – Sodium Dodecyl Sulfate

SF – Serum Free

Strep - Streptavidin

T-ALL – T-cell Acute Lymphoblastic Leukemia

TBS – Tris Buffered Saline

TBS-T – Tris Buffered Saline-Tween

TRE – Tet-response element

UV – Ultra-violet

VBI – Ventral Blood Island

VE – Visceral Endoderm

VE-Cadherin – Vascular Endothelial-Cadherin

VEGF – Vascular Endothelial Growth Factor

Xnr – *Xenopus* nodal related

CHAPTER 1: INTRODUCTION

Animals start their life as a single cell, which undergoes rapid cell division. Even though the daughter cells appear identical in morphology, they are slightly different at the molecular level. These slight differences, inherent in the egg or initiated by outside factors like sperm entry point, are then amplified through cell to cell signaling mechanisms and gene expression networks, which finally differentiate cells to specialized types (reviewed by Rossant and Tam (Rossant and Tam, 2009)).

Although much effort has been put into understanding how a complete organism develops from a single cell, the details of many pathways remain unknown. The transcription factor SCL/TAL-1 is known to be crucial for the development of the blood lineage, but how this induction happens in unclear. Here, I have used the differentiation of pluripotent cells as a model to investigate the role of SCL in early mammalian development in detail.

Formation of mesoderm in the *Xenopus* model

Initial studies on the induction of mesoderm were performed on *Xenopus* embryos by Nieuwkoop (Nieuwkoop, 1985). His work showed that the precursors of definitive endoderm and mesodermal tissues (referred to as *mesendoderm*) are induced in the region where the ectoderm and the endoderm are in contact, the marginal zone. Using explants, he showed that signals from the endoderm induced mesendoderm, even on the animal cap of the embryo, which is normally fated to become ectoderm.

VegT is a vegetal-specific T-box family transcription factor (Stennard et al., 1996), which is necessary for the induction of the mesoderm (Zhang et al., 1998).

Expression of TGFβ family members derriére, Xnr1, Xnr2 and Xnr4 rescue mesoderm formation in VegT depleted embryos (Kofron et al., 1999), demonstrating the involvement of the TGFβ pathway in mesoderm initiation process. Blocking the Activin signal, another TGFβ family member, through expression of a dominant negative receptor, inhibits mesoderm induction (Hemmati-Brivanlou and Melton, 1992). Recently, the mesoderm inducing role of a vegetal TGFβ family member, Vg1, has also been shown (Birsoy et al., 2006).

Mesoderm induction also requires FGF signaling evidenced by the absence of mesoderm in *Xenopus* embryos expressing a dominant negative form of FGF receptor (Amaya et al., 1991).

Mesoderm patterning

After the induction, the dorsal-ventral patterning of the mesoderm is established. Cells in the dorsal end of the embryo give rise to neural tube, notochord and somites, while the ventral cells produce the blood lineage (Dale and Slack, 1987a). Initial insights into the patterning of mesoderm over the dorsal-ventral axis were gained from explant experiments. Spemann and Mangold transplanted cells from the dorsal region of amphibian embryos into other embryos and observed appearance of a second dorsal axis in the recipients. (Spemann and Mangold, 2001) This region was named Spemann's organizer.

Noggin (Smith and Harland, 1992; Smith et al., 1993) and Chordin (Sasai et al., 1994) were cloned from Spemann's organizer region and were shown to dorsalize embryos. In parallel with this work, BMP4 was shown to ventralize embryos when

injected (Dale et al., 1992; Jones et al., 1992). BMP4 could not induce mesoderm. Its effects were limited to mesoderm patterning (Eimon and Harland, 1999; Jones et al., 1996). These observations were unified, when Noggin and Chordin were shown to be inhibitors of BMP signaling (Piccolo et al., 1996; Zimmerman et al., 1996). It became clear that the dorsal and ventral signals were in fact competing factors in the same pathway.

An important feature of the BMP4 signal is dose-dependency. In experiments with *Xenopus* ectoderm, different tissue types formed in response to different levels of BMP4 signal (Wilson et al., 1997). Nuclear factor Smad1 was shown to be the downstream effector of BMP4 in the same study.

At the beginning of development, the *Xenopus* embryo uses maternal transcripts stored in the egg. Damaging these transcripts by UV irradiation causes defects in the formation of dorsal mesoderm (Holwill et al., 1987). Injection of the RNA for a Wnt factor (Xwnt8) into the vegetal side rescues dorsal mesoderm formation in these embryos (Smith and Harland, 1991). This suggests a role for the Wnt signal in dorsalization. In the opposite experiment, inhibition of Wnt signaling, either by overexpression of cadherins or decreasing the level of β-catenin, results in loss of dorsal mesoderm (Heasman et al., 1994). While the dorsal mesoderm markers are lost in these embryos, ventral mesoderm markers are not affected. Wnt11 is shown to be located in the dorsal side of the vegetal endoderm and activate the Wnt pathway to induce dorsal mesoderm (Tao et al., 2005). Rescue of β-catenin depletion phenotype by injection of Noggin shows that Wnt pathway dorsalizes embryo through the inhibition of BMP4 pathway (Wylie et al., 1996).

Further experimentation with explants showed that the dorsal end of the vegetal endoderm induced dorsal mesoderm and ventral vegetal endoderm induced ventral mesoderm, suggesting compartmentalization is not limited to the mesoderm forming marginal zone, but is triggered from the vegetal side of the embryo (Dale and Slack, 1987b). Mesoderm inducing factor Vg1 is enriched on the dorsal half of the vegetal side. Vg1 does not induce the Wnt pathway, but is required for the expression of Chordin (Birsoy et al., 2006). Vg1, therefore, is a candidate that might explain dorsal mesoderm inducing activity of the dorsal vegetal endoderm.

Even though mesoderm induction and patterning might be expected to be separate events, evidence shows the boundaries are not clear-cut. The mesoderm inducing factor Activin acts as a morphogen on tailbud stage embryos and causes formation of different mesodermal tissues (Green et al., 1992). One of the factors that connects Activin, and more generally TGFβ family, signaling between mesoderm induction and patterning is the downstream effector Smad2 (Nakao et al., 1997). VegT, an inducer of TGFβ signaling molecules (Kofron et al., 1999) and Vg1 both activate Smad2 (Birsoy et al., 2006; Lee et al., 2001). Active Smad2, in turn, competes with ventralizing Smad1 signal (Candia et al., 1997).

Mesoderm patterning in the *Xenopus* embryo can be summarized as the competition between Smad-1 inducing BMP family signals (ventral) and Smad-2 inducing TGFβ family signals (dorsal). A study by Schohl and Fagotto visualizes the interplay between these two pathways and the Wnt pathway by immunofluorescent detection of active Smad's and nuclear β-catenin (Schohl and Fagotto, 2002).

Initiation of mesoderm in the mouse model

A major difference between *Xenopus* and mouse mesoderm formation is the role played by maternal transcripts. Zygotic transcription starts much earlier in the mouse embryo compared to the *Xenopus* embryo. Most of the mouse maternal RNA is degraded by the time the embryo reaches the 4-cell stage (reviewed by Zernicka-Goetz et.al. (Zernicka-Goetz et al., 2009)).

Before implantation, there are three distinct tissue types in the mouse embryo: The extraembryonic ectoderm; primitive endoderm, which forms the visceral endoderm and the epiblast or primitive ectoderm, which forms the embryo proper (Gardner, 1985). Between E6.0 and E6.5, a group of epiblast cells on the posterior side of the embryo start moving inward to form the primitive streak (reviewed by Tam and Loebel (Tam et al., 2006)).

A major source for signals that initiate primitive streak formation is the visceral endoderm. In fact, anterior-posterior axis forms in the visceral endoderm before the epiblast (reviewed by Rossant and Tam (Rossant and Tam, 2009)).

The first sign of asymmetry in the visceral endoderm is the thickening of the distal tip (Rivera-Perez et al., 2003). This region corresponds to the expression site of the Hex gene and moves to the anterior-proximal region of the embryo, opposite from the primitive streak initiation site (Thomas et al., 1998). Movement of distal visceral endoderm to the anterior position requires the homeobox transcription factor Otx2 (Perea-Gomez et al., 2001).

Anterior visceral endoderm is responsible for the differentiation of the anterior epiblast to ectodermal lineage (Thomas and Beddington, 1996). This is accomplished

through the inhibition of primitive streak initiating signals emanating from the posterior side of the embryo (Kimura et al., 2000). AVE produces Cerr1 (Cerberus related 1) (Shawlot et al., 1998), homolog of *Xenopus* gene Cerberus, which blocks Nodal, BMP and Wnt pathways (Piccolo et al., 1999); Lefty1 (Oulad-Abdelghani et al., 1998), which inhibits Nodal signaling specifically (Chen and Shen, 2004) and Dkk1, an inhibitor of Wnt signaling (Chen and Shen, 2004).

Like in *Xenopus*, TGFβ family members have been shown to be involved in mouse mesendoderm induction. Expression of Nodal (Varlet et al., 1997) and its co-receptor Cripto (Ding et al., 1998) are stronger on the posterior-proximal side of the embryo at pre-streak stage. Anterior visceral endoderm (Brennan et al., 2001) and the primitive streak (Conlon et al., 1994; Ding et al., 1998) do not form in the absence of Nodal or Cripto. Formation of AVE by Nodal signaling requires the expression of two transcription factors in the visceral endoderm: FoxA2 (HNF3β) and LHX1 (Lim1) (Perea-Gomez et al., 1999). When both factors are removed genetically, expression of the primitive streak genes expands to whole epiblast instead of being limited to the posterior region. This observation underlines the significance of Nodal inhibitor expression from the AVE.

Primitive streak does not form in the absence of BMP4 (Winnier et al., 1995). Some of the knockout embryos can form mesoderm, but this might be due to compensation by BMP2 (Suzuki et al., 1994). The phenotype is more severe in the knockouts of BMP receptor component Bmpr1 (Mishina et al., 1995). Extraembryonic ectoderm, which is adjacent to the proximal epiblast, is a source of BMP4 in the pre-streak embryo (Lawson et al., 1999). When the extraembryonic ectoderm is removed

(Rodriguez et al., 2005) or BMP4 is knocked down (Soares et al., 2005), AVE formation is abnormal, suggesting in addition to its role in primitive streak formation, BMP4 is also required for the patterning of the visceral endoderm.

It is worth noting at this point, that both Nodal (Camus et al., 2006) and BMP (Di-Gregorio et al., 2007) signaling are involved in the maintenance of epiblast pluripotency and the observed effects on mesoderm might be partly due to this earlier function.

Another major signaling pathway shown to be required for mesoderm formation in the *Xenopus* model, Wnt, is also required for mouse primitive streak formation. Wnt3, like Nodal and Cripto, is expressed in the proximal epiblast in the pre-streak stage and embryos lacking Wnt3 do not form a primitive streak (Liu et al., 1999). In these animals, some of the AVE markers are expressed and localize correctly. However, in β-catenin knockout animals, AVE marker expression and migration from distal tip to anterior-proximal region is disrupted (Huelsken et al., 2000). This shows that Wnt signaling, like Nodal and BMP4, is required both for primitive streak initiation and AVE formation. The weaker phenotype in the Wnt3 mutant animals might be due to compensation from other Wnt ligands.

The three signaling pathways (Nodal (TGFβ), BMP4 and Wnt) are interconnected. Two extraembryonic ectoderm proteases, Furin and PACE4, are responsible for initial activation of the Nodal pathway. Work with a Nodal mutant that cannot be cleaved by these proteases showed that active Nodal is required to maintain BMP4 expression in the ExE (Ben-Haim et al., 2006). Culturing the epiblasts in the

absence of ExE and visceral endoderm, but with ectopic BMP4, restores Wnt expression and mesoderm formation. Wnt3, in turn, is necessary for the maintenance of Nodal.

Mesoderm patterning in the mouse

The fates of the epiblast cells are not established before entering the primitive streak. Even though posterior epiblast cells are more likely to contribute to the primitive streak, descendants of labeled cells from most regions of epiblast can be found in mesoderm or endoderm tissues later on (Lawson et al., 1991). However, once the streak is formed, the fates of the cells are determined according to their position on the proximal-distal axis (Kinder et al., 1999; Tam and Beddington, 1987; Wilson and Beddington, 1996). Cells at the proximal end of the streak form the extraembryonic mesoderm, followed by the lateral plate and heart mesoderm lineages. Paraxial mesoderm, notochord and the node, the region corresponding to the Spemann's organizer in the mouse (Beddington, 1994) and the progenitors of the definitive endoderm are at the distal end of the streak. In this sense, organization of the mouse primitive streak resembles that of *Xenopus* marginal zone, with proximal streak corresponding to the ventral and distal streak to the dorsal end.

Similar to the amphibian embryo, Smad2 activation by Nodal signal is required for the formation of distal fates in the primitive streak. Removal of Smad2 or Nodal expression in the primitive streak, results in the loss of distal primitive streak lineage definitive endoderm (Vincent et al., 2003). When one copy of Smad3, the other activator for the Nodal pathway is removed in a Smad2-/- background, this further diminishment of the signal causes the node region to be lost. At minimum signal level, removal of both

Smad2 and 3 in the epiblast, only the most proximal streak lineages, extraembryonic and lateral plate mesoderm are formed (Dunn et al., 2004).

Hematopoiesis

There are two sites of hematopoiesis in the *Xenopus* embryo: The ventral blood island and the dorsal lateral plate (Kau and Turpen, 1983). The VBI is the initial site of hematopoiesis. Cells from this region circulate in the early embryo (Chen and Turpen, 1995). However, the contribution from the VBI is replaced by DLP in time. Therefore, VBI hematopoiesis is considered primitive and DLP hematopoiesis is considered definitive. Transplantation experiments showed that progenitors in both of these regions originate from the ventral marginal zone of the embryo (Turpen et al., 1997).

The first sign of blood in the mouse embryo is the appearance of erythrocytes in the yolk sac. They were first observed by Sabin in chick embryos as sacs of blood cells surrounded by endothelial cells and termed *blood islands* (Sabin, 1920). Yolk sac hematopoietic cells, homologous to *Xenopus* VBI progenitors, descend from the posterior end of the primitive streak (Lawson et al., 1991).

The close association of hematopoietic and endothelial cells in the blood islands, gave rise to the hemangioblast hypothesis (Sabin, 1920). The hemangioblast cell is thought to be the common precursor for both the hematopoietic and endothelial lineage. According to the clonal analysis studies, the hemangioblast is located in the posterior primitive streak and expresses Flk1 surface marker (Huber et al., 2004).

Lately, this simplistic view of the blood island structure (Ferkowicz and Yoder, 2005) and hemangioblast (Ueno and Weissman, 2006), where the hemangioblast migrates

to the yolk sac and differentiates to hematopoietic and endothelial cells that remain together, has been challenged. Images obtained using more recent technologies show that CD41 surface marker expressing erythrocyte cells are first created near the primitive streak and then move into the yolk sac (Ferkowicz et al., 2003). After migration, the cells do not stay in isolated patches (islands), but form a band around the yolk sac.

To determine if the yolk sac blood islands are clonal, Ueno and Weissman co-injected ES cells labeled with different fluorescent markers into blastocysts (Ueno and Weissman, 2006). Blood islands in these embryos contained progenitors with different labels, proving their mixed origin.

The study of the hemangioblast is hampered by the lack of reliable surface marker that identifies the progenitor from other mesodermal cells. The only way to quantify hemangioblasts is an *in vitro* functional assay (Choi et al., 1998). Also, based on recent papers cited here, *in vivo* relevancy of the model is in doubt.

Hematopoiesis also occurs in the embryo proper in the AGM region (Medvinsky and Dzierzak, 1996; Muller et al., 1994). Unlike yolk sac hematopoietic cells, which can only rescue hematopoiesis in irradiated neonate animals (Yoder and Hiatt, 1997); AGM cells can repopulate an irradiated adult animal and thus are considered definitive hematopoietic stem cells (Muller et al., 1994). More careful dissection of the AGM proved the dorsal aorta to be the site of HSC emergence (de Bruijn et al., 2000). Endothelial cells in this region have been shown to give rise to blood cells and are thus termed hemogenic endothelium (Eilken et al., 2009).

Allantoic mesoderm has recently been identified as another source of hematopoiesis (Caprioli et al., 1998).

Molecular biology of blood formation

Signals from the visceral endoderm are needed for hematopoietic induction of the mesoderm. When the VE is removed from the pre-streak or early streak mouse embryos, hematopoiesis is not initiated (Belaoussoff et al., 1998). Furthermore, when anterior epiblast is incubated with the posterior visceral endedorm, ectopic blood is formed. In later studies, Indian Hedgehog has been suggested to be the inductive signal (Dyer et al., 2001). Even though Hedgehog signal is sufficient for the induction of hematopoiesis; it is dispensable *in vivo*, evidenced by blood formation in the embryos lacking Ihh (St-Jacques et al., 1999) or its receptor Smoothened (Zhang et al., 2001).

BMP4, a ventralizing factor in the *Xenopus*, is one of the factors upregulated in response to Ihh (Dyer et al., 2001). However, the study of BMP4 in determination of posterior primitive streak fates is complicated by its earlier function in the formation of the primitive streak. However, both groups who have worked with BMP4 knockout animals noted decreases in posterior primitive streak descendants, like yolk sac blood islands (Lawson et al., 1999; Winnier et al., 1995). Expression of BMP4 inhibitors Noggin (McMahon et al., 1998) and Chordin (Kinder et al., 2001b) from the node supports a model where BMP4 acts as a pro-posterior factor.

In *Xenopus* embryos BMP4 has been shown to induce hematopoietic factors including SCL (Mead et al., 1998), LMO2 (Mead et al., 2001) and GATA2 (Maeno et al., 1996). Expression of SCL or GATA2 can in turn induce hematopoiesis in animal explants (Maeno et al., 1996; Mead et al., 1998). Ets related transcription factor Fli1, a required factor for blood formation, can also induce SCL and GATA2 expression, when ectopically expressed as a fusion protein with the activator VP16 domain (Liu et al.,

2008). SCL, GATA2 and Fli1 were shown to bind to enhancer sites on each other's promoters and form a self-regulating network (Pimanda et al., 2007).

SCL/TAL-1

SCL was first discovered as an oncogene in T-ALL cells and subsequently cloned (Begley et al., 1989; Chen et al., 1990). It is a class B bHLH protein. Class B bHLH proteins, a family that also includes MyoD, can only bind DNA by forming heterodimers with class A bHLH proteins, like E47 or E12, products of the E2A gene.

A number of studies in different models show that SCL is not just an oncogene, but is the master regulator of hematopoiesis. The gene is expressed in hematopoietic, endothelial and some neural progenitors during mouse development (Elefanty et al., 1999). Knockout mice die of anemia from E8.5 to E10.5 (Elefanty et al., 1999; Robb et al., 1995; Shivdasani et al., 1995). The SCL-/- cells in chimeric animals contribute to all tissues except hematopoietic (Porcher et al., 1996; Robb et al., 1996) and are unable to produce hematopoietic cells when differentiated *in vitro* (Porcher et al., 1996). Additionally, ectopic expression of SCL in zebrafish *cloche* mutants rescues blood and endothelial defects (Liao et al., 1998). Vascular network formation is also disrupted in SCL-/- mice and this phenotype is not secondary to lack of hematopoiesis (Visvader et al., 1998). In transgenic animals where blood formation is rescued through expression of SCL under the control of the hematopoietic-specific GATA1 promoter, the yolk sac vascular network still does not form, suggesting this to be an endothelial-specific SCL function.

Using a lacZ/knock-in strategy SCL expression has been reported to start on E7.5 in extraembryonic mesoderm of the mouse embryos (Elefanty et al., 1999). The expression is later observed in the endothelium and the central nervous system. In this study, the researchers mostly reported expression data from embryos that have both their SCL alleles replaced with lacZ, because the expression is too low to detect in heterozygous embryos. Thus, the expression data obtained from these embryos may not represent the patterns in wild-type embryos.

In adult human hematopoietic tissues, SCL is expressed in erythrocyte, megakaryocyte and basophilic lineages (Mouthon et al., 1993; Pulford et al., 1995). Overexpression of the protein in MEL or K562 cells (leukemia cell lines that can differentiate to erythrocyte or other lineages) resulted in increased differentiation towards the erythroid lineage (Aplan et al., 1992).

Hall and colleagues have shown the role of SCL in erythrocyte and megakaryocyte maturation, through a conditional deletion of the gene in the adult mice (Hall et al., 2003). In response to SCL deletion, progenitor cells from both lineages were lost, followed by a loss of mature cells. However, a long term follow-up study from the same group also showed that after an initial drop in erythrocyte counts, mice recovered erythropoiesis and reached a state of mild anemia (Hall et al., 2005). They concluded that SCL is important but not essential for adult erythropoiesis.

The role for SCL for the emergence and the maintenance of the HSC compartment has been studied by conditional deletion of the gene. Deletion of SCL in Tie2 expressing cells did not affect the formation of the HSCs (Schlaeger et al., 2005). Conditional deletion of SCL in the adult bone marrow resulted in a defect in short term

repopulating HSCs, while long term repopulating HSCs remained unaffected (Curtis et al., 2004). On the other hand, ectopic expression of SCL increased the repopulating activity of the LTR-HSCs (Reynaud et al., 2005) and HSCs are less competitive after SCL knockdown (Lacombe et al.). The observed conflict could be due to compensation by Lyl1, a related bHLH factor. Removal of both SCL and Lyl1 resulted in significant loss of HSCs (Souroullas et al., 2009).

SCL complex

Apart from erythropoiesis- and megakaryopoiesis-specific observations, studies in cancer cell lines also provided insight on mechanism of SCL function (Krosl et al., 1998; Lahlil et al., 2004; Vitelli et al., 2000).

SCL was shown to interact with LMO2, in two independent studies using hematopoietic cell lines (Valge-Archer et al., 1994; Wadman et al., 1994). Later on, one of the groups showed that this interaction is not limited to SCL and LMO2, but the complex also includes GATA1, E2A proteins and LDB1 (Wadman et al., 1997). GATA2 can also bind to LMO2 (Osada et al., 1995) and while it is in the complex in multipotent hematopoietic progenitors, GATA1 takes its place after commitment to the erythrocyte lineage (Anguita et al., 2004).

Similar to SCL, LMO2 (Lim only 2) was discovered as an aberrantly expressed gene in T cell leukemias (Boehm et al., 1991; Royer-Pokora et al., 1991). Like other members of its family, it contains two LIM domains, which are known to be important in forming protein-protein interactions (Boehm et al., 1991). The protein also has two

transactivation domains and the LIM domains can act as transcriptional repressors (Mao et al., 1997).

LMO2 was shown to act together with SCL in leukemia (Larson et al., 1996) and erythropoiesis (Valge-Archer et al., 1994). Overexpression studies suggested a role for LMO2 and one of its binding partners, Ldb1, in keeping early erythrocyte progenitors undifferentiated (Visvader et al., 1997). The similarity of the LMO2 knockout phenotype to that of SCL (death with anemia around E9-10 (Warren et al., 1994)) and lack of hematopoiesis from *in vitro* differentiated LMO2-/- cells (Yamada et al., 1998) suggest that the two proteins might also work together in early development.

GATA proteins were discovered by virtue of their binding conserved sites in the β-globin locus in erythrocytes (Tsai et al., 1989). GATA1 and GATA2 were shown to be expressed in the ventral marginal zone of *Xenopus* embryos and to mark hematopoiesis (Kelley et al., 1994). In mice, loss of GATA1 results in a defect in erythropoiesis (Pevny et al., 1991), while GATA2 is crucial for the proliferation of early hematopoietic progenitors (Tsai et al., 1994; Tsai and Orkin, 1997).

DNA sequences bound by the SCL complex contain consensus sequences for an E-box site for E2A proteins and a GATA binding site (Wadman et al., 1997). However, not all targets of SCL have this binding sequence in their promoters (Lecuyer et al., 2002).

SCL in early development

Different roles, targets and biochemical interactions in different cell types are common for transcription factors. Therefore, data gathered from leukemic cell lines or

adult hematopoietic cells may not be applicable to an earlier, mesoderm stage function of SCL. Studies directed at this specific developmental stage have been performed mainly in non-mammalian models, zebrafish and *Xenopus*.

Ectopic expression of SCL in zebrafish (Gering et al., 1998) and *Xenopus* (Mead et al., 1998) embryos increases hematopoietic and endothelial progenitors by reprogramming pronephric and somitic mesoderm. In zebrafish co-expression or induction of LMO2 was proposed to be necessary for the reprogramming activity (Gering et al., 2003).

ES cell differentiation as a model of early mouse development

Mammalian embryos are not easy to manipulate. Therefore discovery experiments in the mouse are often impractical, if not impossible and even the confirmation of findings from non-mammalian models can be difficult. To overcome limitations inherent in the small size of the early mouse embryo and primitive streak, *in vitro* differentiation of ES cells is being used increasingly in developmental biology.

Mouse ES cells, which have potential to differentiate into lineages of the three germ layers (Bradley et al., 1984) were first cultured in 1981 by Evans and Kaufman from the inner cell mass of embryos (Evans and Kaufman, 1981). They can be maintained in an undifferentiated state indefinitely and induced to differentiate through manipulation of culture conditions. This allows production of ample material for use in a number of molecular biology techniques, like microarrays or biochemistry; as well as fine manipulation of culture conditions for analysis of extracellular and genomes for intracellular mechanisms.

One common method of differentiating ES cells is *embryoid body aggregation*. When ES cells are incubated without attachment (in semisolid medium or with constant shaking), they aggreagate and form three dimensional structures called embryoid bodies (EB) (Doetschman et al., 1985). A number of embryonic lineages have been shown to form in these structures.

Hematopoiesis in EBs and embryos has been compared by Keller and colleagues and similar time-frames and cell types are shown to be active in both (Keller et al., 1993). Also, the hypothetical mouse hemangioblast (Sabin, 1920), was initially identified *in vitro* using embroid bodies (EB) (Choi et al., 1998). Culture conditions developed in this study led to the discovery of the *in vivo* hemangioblast (Huber et al., 2004). Using a cell line in which GFP is expressed under the control of the Brachyury promoter, it has been shown that the hemangioblastic and hematopoietic precursors emerge in the Brachyury-expressing population and later begin expressing Flk1 (VEGF-Receptor-2) protein (Fehling et al., 2003).

Most ES cell differentiation studies have been performed in FBS-containing media. The growth factors in the FBS are largely unknown and their concentrations change between batches, which hampers comparability of data from different groups. Recently, there has been a developmental biology-inspired effort to reveal the identities of serum growth factors and to grow cells in chemically defined media.

Embryoid bodies, which were incubated with Activin A or BMP4 in a chemically defined medium, formed anterior or posterior mesodermal structures respectively (Johansson and Wiles, 1995). High levels of Activin A treatment also induces definitive endoderm formation (Kubo et al., 2004). Nodal is the active TGFβ member in the early

mouse embryo, but it signals through the Activin receptors and downstream effectors (Gu et al., 1998; Song et al., 1999). *In vitro* Activin treatment is thought to be mimicking Nodal's *in vivo* activity.

A protocol for production of blood lineage cells from the ES cells, based on sequential treatment of the cells with BMP4, Activin A, bFGF and VEGF has been published recently (Pearson et al., 2008). The group proposed a model in which BMP4 is required for the induction of mesoderm based on the expression of GFP under the control of Brachyury promoter. Then Activin and bFGF treatment induces the hemangioblast, evidenced by co-expression of Flk1 and Brachyury (Fehling et al., 2003). Finally, VEGF treatment helps differentiation of blood lineage from the hemangioblast. A second group came to the same conclusion about the roles of BMP4 and VEGF, but did not observe a strong effect of Activin treatment (Park et al., 2004).

The discovery of human ES cells (Thomson et al., 1998) and recent success in derivation of pluripotent cells from somatic cells (Takahashi and Yamanaka, 2006) increased the interest in directed differentiation of pluripotent cells to specific lineages with the hope that one day patient-specific tissues can be produced in the laboratory. Using the knowledge gained from developmental biology, protocols for efficient derivation of a number of tissues, including neural (Chambers et al., 2009), cardiac (Yang et al., 2008), skeletal muscle (Darabi et al., 2008) and pancreatic mesoderm (D'Amour et al., 2006) have been described. Some of these studies are close to clinical trials.

CHAPTER 2: MATERIALS AND METHODS

Cell lines and Cloning

Creation of engineered ES lines using Ainv15, A2.lox.cre or Zx1 cell lines and plox or p2lox targeting plasmids has been described in detail elsewhere (Iacovino et al., 2009) (Fig 2.1). Briefly, a locus containing a TRE sequence for inducible expression, two lox sites that do not recombine with each other, Cre cDNA sequence and a Neo sequence devoid of an ATG is inserted into the X chromosome of targeting cells. (A2lox.cre or Zx1). The p2lox plasmid contains the same lox sites, and the gene of interest. Through Cre mediated recombination, the gene of interest is inserted after the TRE promoter and the Neo gene is fixed with the addition of the PGK promoter and an ATG. Neo can be used for selection of successfully targeted cells.

iSCL, iLMO2, iGATA2. iSCL-LMO2 and iSCL-LMO2-GATA2 cell lines were created using Ainv15 parental cell line and plox targeting plasmid by Dr. Rita Perlingeiro.

Human SCL cDNA, a gift from Dr. Stuart Orkin, was cloned into the p2lox plasmid, by digesting with EcoRI and SmaI from plox-SCL and ligating into EcoRI and blunted NotI sites.

VP16 and Engrailed constructs were amplified by PCR with a linker containing the first few bases of SCL coding sequence until the NotI digestion site.

VP16-SCL-F: GAATTCACCATGGCCCCCCCGACCG

VP16-SCL-R: GCGGCCGCTCCGTCATGTCGACCCCACCGTACTCGTC

Eng-SCL-F: GAATTCACCATGGCCCTGGAGGATCG

Figure 2.1

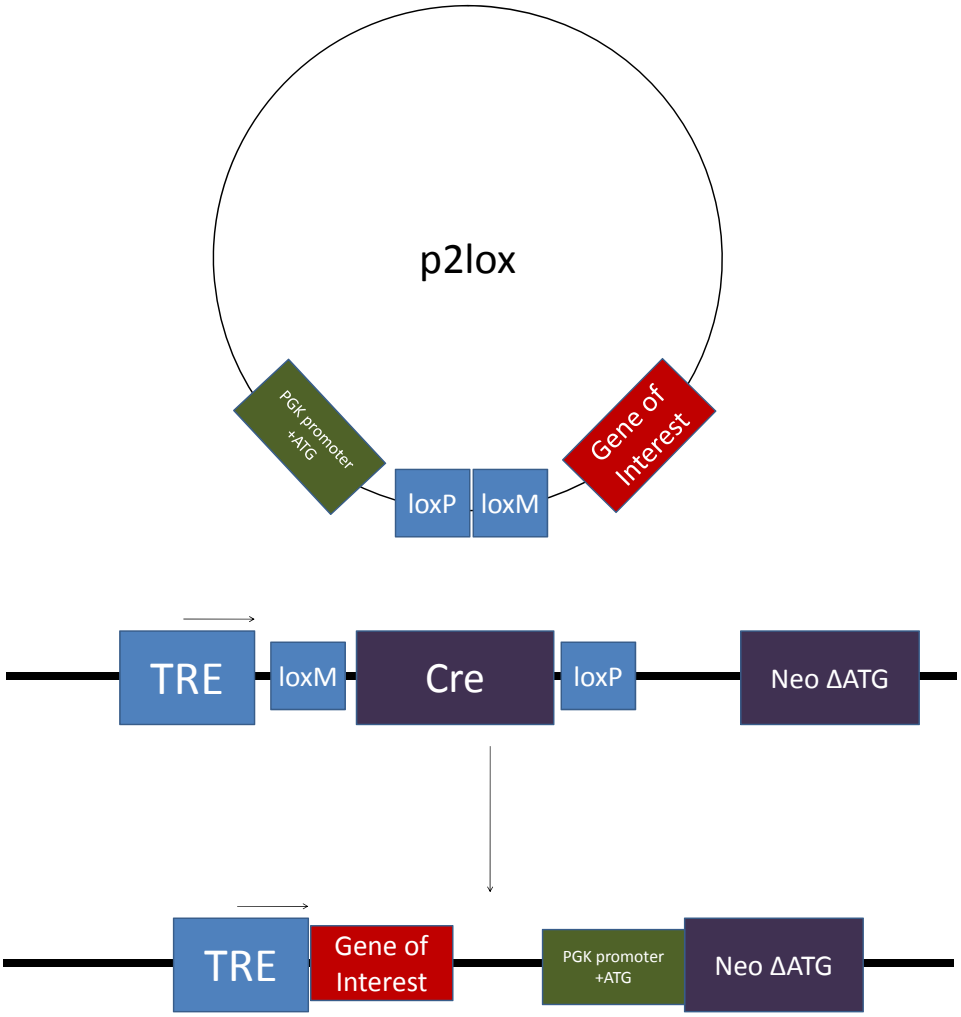


Figure 2.1: Representation of the p2lox plasmid and the HPRT locus of A2lox.cre cells where the targeting sequence has been inserted. Cre expression is induced with Dox before targeting and the Cre cDNA is removed after lox recombination. TRE: Tet response element. Neo ΔATG: Neo sequence without a start codon.

Eng-SCL-R: GCGGCCGCTCCGTCATCTCGAGCAGAGCAGATTTCTCTGG

Then, these amplicons were inserted into p2lox-SCL after EcoRI-NotI digestion, to create p2lox-VP16SCL and p2lox-EngSCL plasmids. These plasmids were used to create iVP16-SCL and iEng-SCL cell lines.

iSCLF238G and iSCLRERAAA constructs were cloned into the p2lox plasmid by PCR amplification with primers designed for site directed mutagenesis and EcoRI and NotI digestion.

SCLF238G-Int-F: CCATGAAGTACATCAATGGCCTGGCC

SCLF238G-Int-R: GTAACTTGGCCAGGCCATTGATGTAC

SCLRERAAA-Int-F: TTCACCAACAGCGCAGCTGCATGGAC

SCLRERAAA-Int-R: CTGCTGCCTCCATGCAGCTGCGCTGT

SCL-Ext-F: CTCGAGATGACGGAGCGGCCGCCGAGC

SCL-Ext-R: GAATTCTCACCGGGGGCCAGCCCCATC

Avi-SCL-IRES-BirA construct was synthesized by Geneocopoeia company and subcloned into the p2lox plasmid for the generation of Zx1.Avi-SCL.BirA cells.

Plasmids were inserted into the cells either by electroporation of 30μg of DNA (240V, 500μFad) or transfection of 1μg of plasmid DNA using Fugene 6 (Roche # 11814443001) into ~1-2X10^6 cells. In the case of electroporation, the cells were plated onto G418-resistant mitomycin treated MEFs (Millipore #PMEF-N). When Ainv15 cells were used, a Cre expressing plasmid was co-electroporated or transfected. A2.lox.cre and Zx1 cells have Cre cDNA under the control of the Dox inducible promoter. These cells were induced for 24 hours before electroporation or transfection. In both protocols cells were allowed to recover for 24 hours in the ES medium and then G418 selection started

(300µg/ml, Invitrogen 10131-035). 7 to 10 days later single ES colonies were picked (6 clones per cell line, if available), trypsinized and plated on MEFs in 12 well plates. The cells were passaged when they became confluent and frozen as P0 vials after 2 passages.

Maintenance of ES cells

Undifferentiated ES cells were maintained on inactivated MEFs in ES Medium containing Knockout DMEM (Invitrogen #10829-018), 15% ES-qualified FBS, Gluta/max (Invitrogen #35050-061), P/S (Invitrogen #15140-122), NEAA (Invitrogen #11140-050), β-mercaptoethanol (Gibco #21985-023) and LIF (5000 units/ml Millipore #ESG1107). Cells were fed every day and passaged every two days or when confluent.

Differentiation of ES cells as EBs

For EB differentiation, ES cells were passaged by treatment with 0.25% Trypsin-EDTA. After the removal of trypsin, cells were replated in EB differentiation medium (IMDM (Invitrogen #12440-053), 15% EB-qualified FBS, Gluta/max, P/S, Transferrin (200 µg/ml), Ascorbic Acid and MTG) and incubated for 30-45 minutes to remove MEFs.

For hanging drops, cells were diluted to a concentration of 10000 cells/ml in EB-differentiation medium. Then, they were plated on a 15 cm untreated bacterial Petri dish as 10µl droplets and incubated upside down for two days. On day 2, EBs were removed from Petri dishes by washing with Ca/Mg-free PBS, replated in 6 or 10 cm petri dishes in EB-diff and placed on an orbital shaker at 60 rpm/minute. Unless noted otherwise, half of the medium was removed from the plate every two days and replaced with fresh EB-diff medium.

For large scale EB preparation, after MEF removal ES cells were incubated in 15 cm untreated Petri dishes at 10000 cells/ml density on the orbital shaker. EBs were fed every two days.

Differentiation of ES cells in monolayer

For monolayer differentiation in serum, 15000 cells were plated per well of a 6-well Corning plate in EB-diff medium. Differentiating cells were fed every day.

For serum-free monolayer differentiation, Corning plates were coated with Growth factor reduced matrigel (BD Biosciences #356234) for 1 hour. After trypsinization and MEF removal, ES cells were plated in ES medium for attachment (15000 cells/well of a 6-well plate or 7500 cells/well of a 12-well plate). The next day ES medium was replaced with serum free differentiation (SF-diff) medium (mTESR medium without growth factors but with added P/S, Gluta/max, Transferrin, Ascorbic acid, MTG). Cells were fed every day with SF-diff medium.

Hematopoietic CFC Assay

Hematopoietic CFC assays were performed in MCM3434 medium (Stem Cell Technologies). 50000 cells were resuspended in 150µl of EB-diff and plated in 1.35ml of MCM3434. EryP colonies were counted 6 days after plating, more differentiated colonies were counted 10 days after plating.

Cardiac beating assay

Single EBs were removed from culture on day 5 and plated in gelatinized wells of a 96 well flat-bottom tissue culture plate in EB-diff. EBs attached and started to spread

overnight. Some beating could be seen after 24 hours. At 48 hours beating was more intense. EBs were scored as “beating” or “not beating”.

Cytospins

Cells were spun onto slides at 1000rpm for 5 min. Slides were then stained with HEMA3 kit (Fisher #22-122-911) according to manufacturer’s instructions and mounted.

FACS Analysis

Between 10^5-$5X10^5$ cells were stained using fluorescently labeled antibodies. Cells were kept in 1:200 dilution of antibodies in staining solution (PBS with 3%FBS) for 25-30 min, followed by one wash in staining medium. After the wash cells were resuspended in cold analysis solution (staining solution with PI), filtered and analyzed using a BD FACSAria instrument with 488nm and 633nm lasers.

Real Time PCR

RNA was isolated using Trizol and chloroform extraction. After resuspension cDNA was produced using a Thermoscript kit (Invitrogen) at 60°C for 1hr.

Taqman probes were used for Real Time PCR. Reactions were run on ABI7500 or 7900 machines.

Microarray Analysis

Day 2.5 EBs were induced for 6 hours with Dox and collected in Trizol. Isolated RNA was processed by the UTSW microarray core facility and hybridized to Illumina bead chips. Data analysis was performed using BeadStudio software.

Immunoprecipitation

EBs were washed with PBS, trypsinized and washed again. Protein was extracted in modified RIPA buffer (50mM Tris-HCL pH:7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-Deoxycholate with fresh protease inhibitors (Roche #11836153001)) for 1 hour on a rotator at 4°C. Lysate was centrifuged at 20000g for 30 minutes at 4°C to remove cellular debris. For treatment with Agarose-Strep beads (Sigma #85881), lysate was precleared with Sepharose-Protein A beads (GE Healthcare #17-0780-01) for 1 hour at 4°C. The beads were blocked with 1% BSA for 1 hour at 4°C. Lysate was not precleared and beads were not blocked, when magnetic Dynabeads were used (Invitrogen #656-01). Lysate was incubated with beads (2µl of Sepharose or 1µl of Dynabeads per mg of total protein) for 1 hour at 4°C. After incubation, the beads were removed (by gravity for Strep beads, magnets for Dynabeads) and washed 6 times with modified RIPA buffer. The beads were then washed in the TEV protease reaction buffer (50mM Tris-HCl, 0.5mM EDTA, 1 mM DTT) overnight. To cleave SCL and bound proteins, the beads were treated with TEV protease in the reaction buffer overnight at 4°C (Invitrogen #12575-015).

Silver staining and Mass Spectrometry

SDS-PAGE gels were stained using a silver staining kit (Pierce #24600) according to the instructions from the manufacturer.

For mass spectrometry proteins were digested in solution with sequence grade trypsin (Promega #V5111) overnight at 37°C. Mass Spectrometry analysis was performed by the Proteomics Core at the University of Minnesota.

Western Blotting

Protein was extracted by boiling EBs in the SDS loading dye for 15 min. After cooling, samples were run on 10% SDS-PAGE gel for 2 hours at 100V. Proteins were transferred onto PVDF membrane at 120mA for 2 hrs in a wet blotting setup. The membrane was blocked 1 hr at RT with 5% milk. Primary antibody (BTL73, a gift from Dr. Karen Pulford) was used in 1:100 dilution in 5% milk for 1hr at 37°C, washed 6 times with TBS-T. Secondary antibody (Donkey anti Mouse- HRP, Santa Cruz) was used in 1:2000 dilution in 5% milk for 1 hr at RT, washed 6 times with TBS-T. Bands were visualized using ECL (Pierce #32109), Pico (Pierce #34079) or Femto kits (Pierce #34094).

CHAPTER 3: THE ROLE OF SCL IN MESODERM PATTERNING

Introduction

In mammalian embryos, the initiation of the development of the mesoderm and the definitive endoderm, commonly referred to as the mesendoderm, is marked by the formation of the primitive streak. Fate mapping studies show that cells at different points along the proximal-distal and anterior-posterior axis of the streak map to different tissues in the adult animal (Kinder et al., 1999; Tam and Beddington, 1987; Wilson and Beddington, 1996). Proximal-posterior primitive streak cells form the extraembryonic mesoderm and the lateral plate mesoderm, which later forms the hematopoietic and endothelial tissues; while the cells located distally form the paraxial mesoderm, the notochord and the node. The intensity of the BMP4 signaling, regulated by the secretion of BMP4 at the proximal end and the secretion of the BMP inhibitors Noggin and Chordin from the distal end of the primitive streak, plays a significant role in determining the fate of the cell (Wilson et al., 1997).

SCL is one of the transcription factors induced by BMP4 (Mead et al., 1998) and was shown to be a critical factor in hematopoietic development in the *Xenopus* (Mead et al., 1998), zebrafish (Gering et al., 1998) and mouse (Elefanty et al., 1999; Robb et al., 1995; Shivdasani et al., 1995) models. The loss of SCL function results in the loss of blood formation, while ectopic SCL expression increases blood forming potential. However, it cannot be inferred from the data whether SCL acts (a) on the mesoderm patterning process, (b) on the committed lateral plate mesoderm cells that are

differentiating towards hematopoietic lineage or (c) on the committed hematopoietic progenitors.

The only previous time course assay on the role of SCL was performed in ES cells cultured on OP9 stromal cells, where SCL expression was absent but could be turned on using a Tamoxifen inducible Cre (Endoh et al., 2002). Turning on SCL expression until day 4 of differentiation on stromal cells rescued hematopoiesis. Only the expression of the Flk1 and VE-Cadherin markers were followed, thus it is not clear which embryonic stage day 4 corresponds to in this system, but a general conclusion of the paper is that SCL is necessary early in the process.

In order to perform a pulse time course assay and analyze SCL function at the specific relevant time points, an ES cell line was engineered to express SCL under the control of a Dox-inducible promoter, which made it possible to turn the expression on and off quickly. I determined the stages of EB differentiation that correspond to the mesoderm induction, patterning and hematopoietic differentiation stages of embryonic development. I induced ectopic SCL expression for 24 hour periods in different samples over a time course and observed an increase in hematopoietic output only in response to the expression during the mesoderm patterning stage. Finally, I showed that SCL increases hematopoietic progenitors by changing the fate of non-hematopoietic mesodermal lineages.

Results

a. Differentiation of wild type EBs

In order to place the stages of differentiation on a timeline in the EB differentiation model, I first analyzed the differentiation of wild type (E14) and uninduced iSCL ES cells using qRT-PCR and FACS.

For qRT-PCR, EBs were grown for 6 days and RNA was collected every 12 hours. E14 and uninduced iSCL EBs start expressing Brachyury, which is a marker of early, unpatterned mesoderm (Fehling et al., 2003; Wilkinson et al., 1990), around day 2; with a peak on day 3. After day 3, Brachyury expression decreases as the mesoderm is patterned to downstream lineages; e.g. blood or cardiac muscle (Fig 3.1A). SCL expression initiates after Brachyury, around day 2.5 and peaks on day 4 (Fig 3.1B). After day 4, SCL expression decreases, but does not disappear completely. This is due to the expression of SCL in the patterned mesoderm and emerging hematopoietic and endothelial cells.

The PDGFRα surface marker is expressed on the early primitive streak cells of murine embryos (Orr-Urtreger et al., 1992). After mesoderm patterning, it is limited to the paraxial mesoderm. Flk1, a receptor for VEGF, is often used as a marker of endothelial cells. However, it also has been shown to be expressed in the early mesoderm and later in the lateral plate mesoderm, precursor of the hematopoietic and endothelial lineages (Kataoka et al., 1997; Nishikawa et al., 1998). $Flk1^+$ cells have also been shown to have cardiac lineage potential (Iida et al., 2005).

PDGFRα expression initiated around day 3 in my experiments. On day 4, the cells progressed to the $Flk1^+$/ $PDGFR\alpha^+$ (DP) state, with small amount of single positives.

Figure 3.1

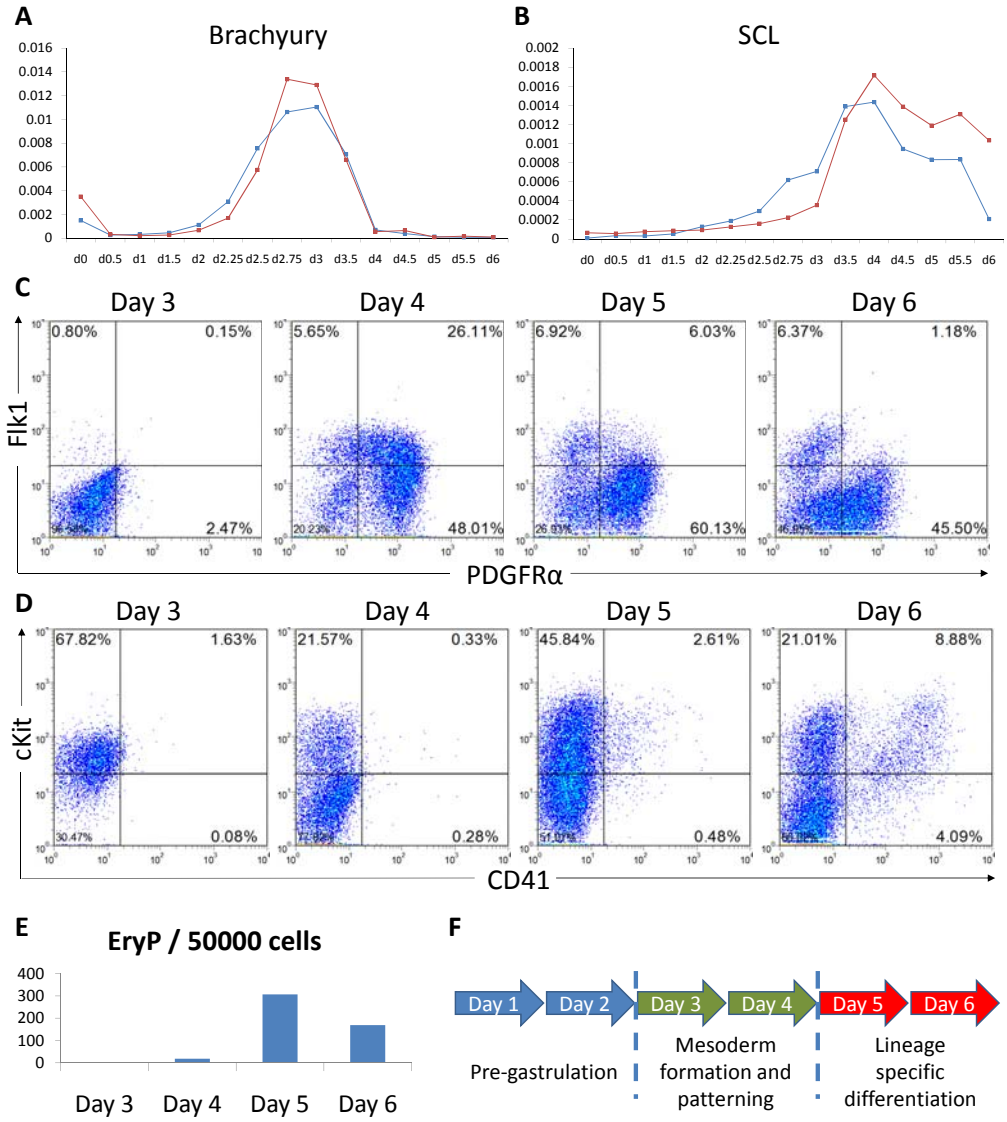


Figure 3.1: Analysis of EB differentiation with wild-type (E14) cells. Expression of Brachyury **(A)** and SCL **(B)** shown over a time course of 6 days. Blue line represents the E14 cells, red line represents the uninduced iSCL cells. **C)** Expression of Flk1 and PDGFRα over time course. **D)** Expression of cKit and CD41 markers over a time course. **E)** Number of EryP colonies obtained per 50000 E14 cells on different days of differentiation. **F)** Schematic representation of EB development

On days 5 and 6 the percentage of the DP cells decreased and better defined $Flk1^+$ or $PDGFR^+$ cell populations emerged (Fig 3.1C).

Comparison of SCL-/- and wild type cells during *in vitro* differentiation, revealed CD41 to be the earliest marker of hematopoiesis (Mikkola et al., 2003). This marker was expressed in the EBs at a low level on day 4 (Fig 3.1D). Another marker of hematopoiesis, CD45 followed CD41 starting on day 5. Accordingly, the first colony forming cells were also obtained from the day 4 EBs. The colony forming activity was limited to the EryP lineage at this point. The number of EryP colony-forming cells peaked on day 5 (Fig 3.1E). More definitive colonies also emerged on this day, with an increase on day 6, the final point of my analysis.

Based on these data, I concluded that in this system mesoderm starts to form on day 2 of differentiation and is patterned until day 4. Terminal differentiation towards mesoderm lineages continue on days 5 and 6 (Fig 3.1F).

b. Ectopic SCL expression increases the hematopoietic output

Ectopic expression studies in model organisms showed that SCL would have a positive effect on the hematopoietic output (Gering et al., 1998; Mead et al., 1998).

I first analyzed the effect of continuous SCL expression from day 2 on. The resulting increase in the ratio of cells expressing hematopoietic surface markers (CD41, CD45) (Fig 3.2A-D) and the number of CFCs per 50000 EB cells was significantly increased, in line with expectations based on the observations in non-mammalian models (Fig 3.2E).

Figure 3.2

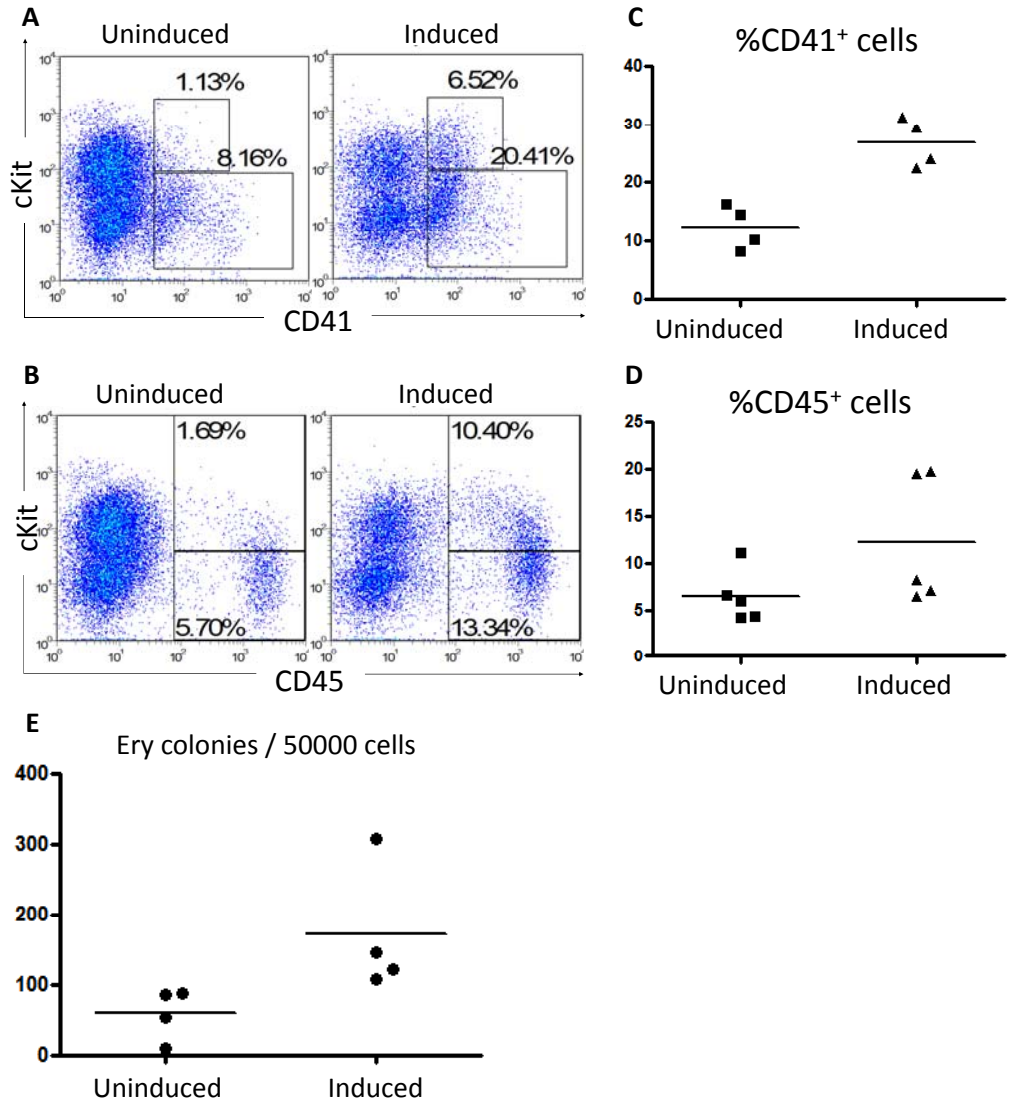


Figure 3.2: Change in the expression of surface markers CD41(**A**) and CD45(**B**) in response to continuous Dox induction from representative experiments. Summary of FACS data CD41 (**C**) and CD45 (**D**) and colony data (**E**) from repeated experiments.

Figure 3.3

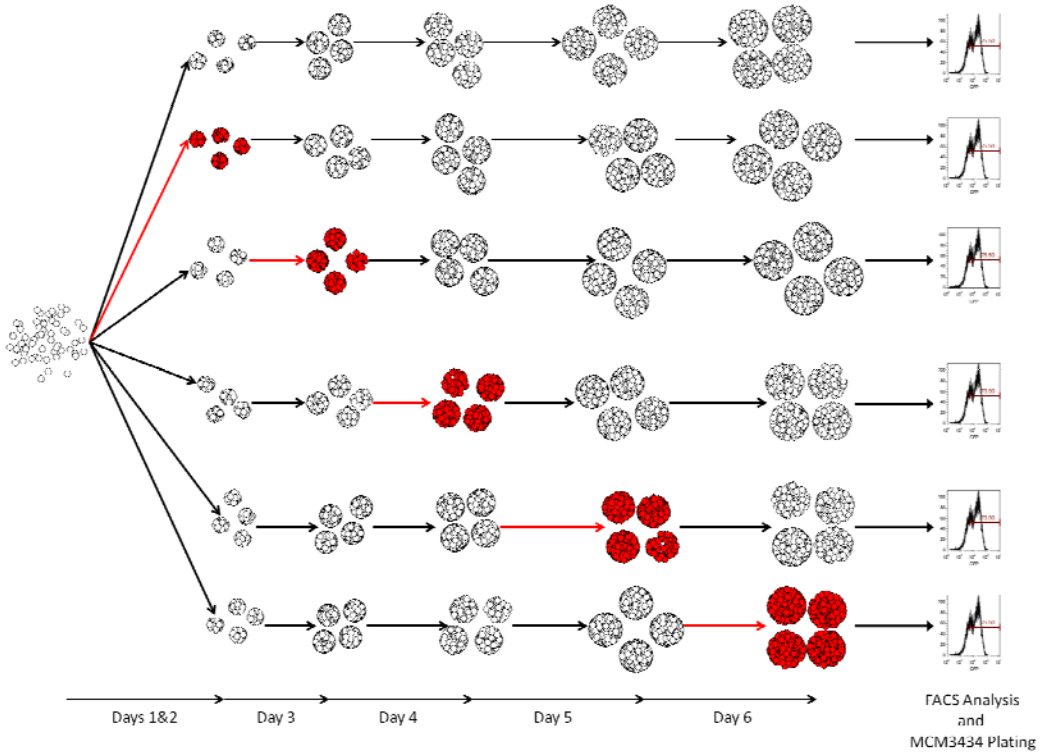


Figure 3.3: Schematic represantation of the 24hr time course experiment. A different sample is induced (red) each day and all samples are analyzed at the end of the experiment on day 6.

c. The effect of SCL is limited to the mesoderm patterning stage

The mechanistic details of SCL's pro-hematopoietic effect are largely unknown. The fact that in the Cre reversion study mentioned above SCL had to be rescued in the knockout cells before the day 4, suggests a specifically timed rather than a general effect (Endoh et al., 2002).

I hypothesized that SCL has a specific window of activity during the EB differentiation. To test this hypothesis I designed an experiment to look for this specific window by pulsed induction at different time points (Fig 3.3). Six differentiation cultures were started. One of the cultures was kept uninduced, one was induced for the first 2 days of differentiation, while the cells were still in hanging drops, and one was induced on each of days 3, 4, 5 and 6 for 24 hours. At the end of each day, medium from all cultures was removed and all medium from uninduced plates was pooled and filtered. Fresh medium was added to compensate for removed Dox containing medium and EBs were replated in this mix. One of the yet uninduced plates was induced for the next 24 hours. At the end of 6 days all EBs were trypsinized and analyzed.

The strongest increase in hematopoiesis was seen in the plates induced on day 4 of differentiation, with weaker effects on days 3 and 5 (Fig 3.4A-E). According to the data from section a, this window corresponds to the mesoderm patterning stage.

d. Ectopic SCL expression increases hematopoietic output by patterning mesoderm

After determining the mesoderm patterning stage as the window of activity for SCL, I hypothesized that it changes the fate of the early mesoderm progenitors. This model is supported by the findings of an overexpression study in zebrafish (Gering et al., 1998) but contradicted by the findings of an ES cell differentiation study (Endoh et al.,

Figure 3.4

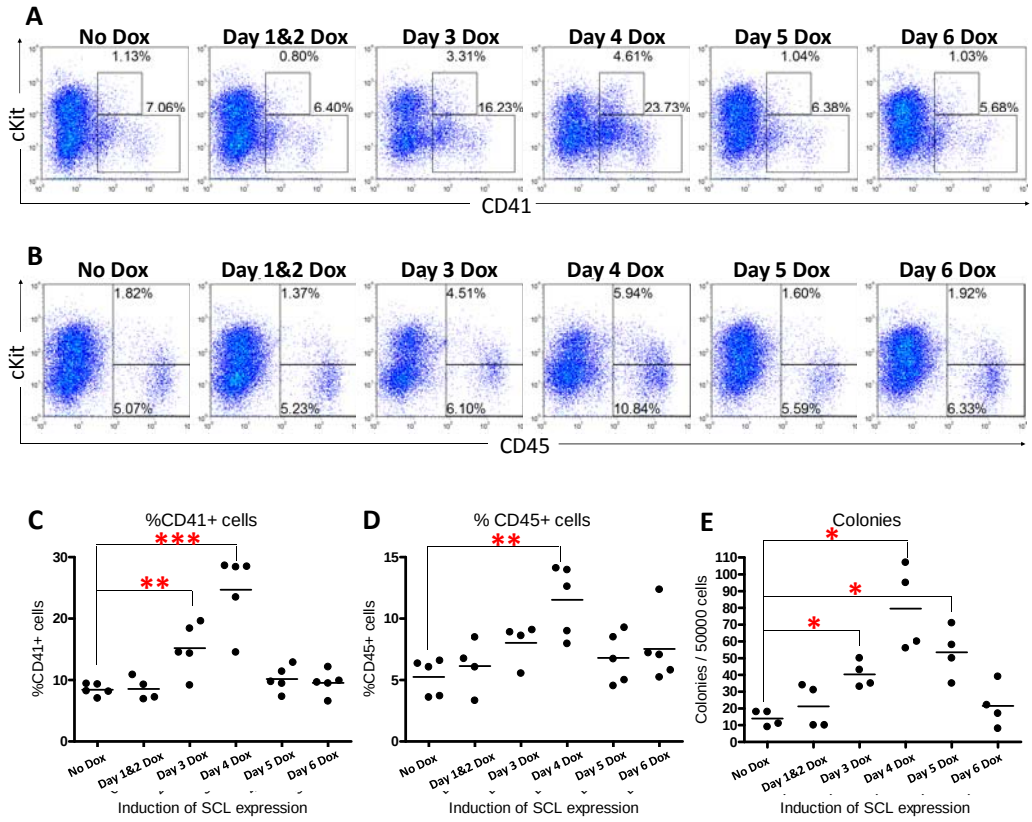


Figure 3.4: Data from the SCL 24 hr time course experiments. Surface phenotype of differentiated iSCL EBs with cKit/CD41(A) and cKit/CD45(B) stainings from representative experiments. Percentage of $CD41^{+}$(C) and $CD45^{+}$(D) obtained at repeated experiments. Data analyzed using students t-test. **:p<0.01, ***:p<0.005. E) Number of colonies obtained at repeated experiments. Data analyzed using Mann-Whitney test, *:p<0.05

2002). To test the hypothesis, I induced iSCL EBs on days 3 and 4 of differentiation for 48 hours and compared the amount of non-hematopoietic mesoderm lineages produced in the uninduced and induced cultures.

The ectopic expression of SCL did not change the level of Oct4 or Brachyury expression in the EBs (Fig 3.5A-B). This shows that SCL did not induce differentiation towards mesoderm under these conditions. However, FACS analysis showed that in the induced EBs, PDGFRα^+ population, which marks the paraxial mesoderm (Kataoka et al., 1997), decreased significantly, while the Flk1$^+$ population, which marks the lateral plate mesoderm increased (Fig 3.5C).

BL-CFC, the counterpart of the hemangioblast in the EBs (Choi et al., 1998), represents the lateral plate mesoderm. In order to confirm the FACS data showing an increase in the lateral plate mesoderm compartment in response to SCL induction, I performed BL-CFC assays on day 3 of the EB differentiation, the time when BL-CFC numbers are closest to their peak. I observed an increase in the number of BL-CFCs after SCL induction (Fig 3.5D).

Cardiac tissue can initiate autonomous beating in culture. By plating day 5 iSCL EBs from uninduced and induced plates and scoring them for beating, I showed that the cardiac mesoderm differentiation was diminished in response to ectopic SCL expression (Fig 3.5E). Results of this functional experiment were confirmed by the more sensitive qRT-PCR analysis. A marker of cardiac differentiation, Nkx2.5, was downregulated in the induced samples (Fig 3.5F).

I tested the expression of the skeletal muscle markers to assess the level of the paraxial mesoderm differentiation. However, the levels were too low to derive a

Figure 3.5

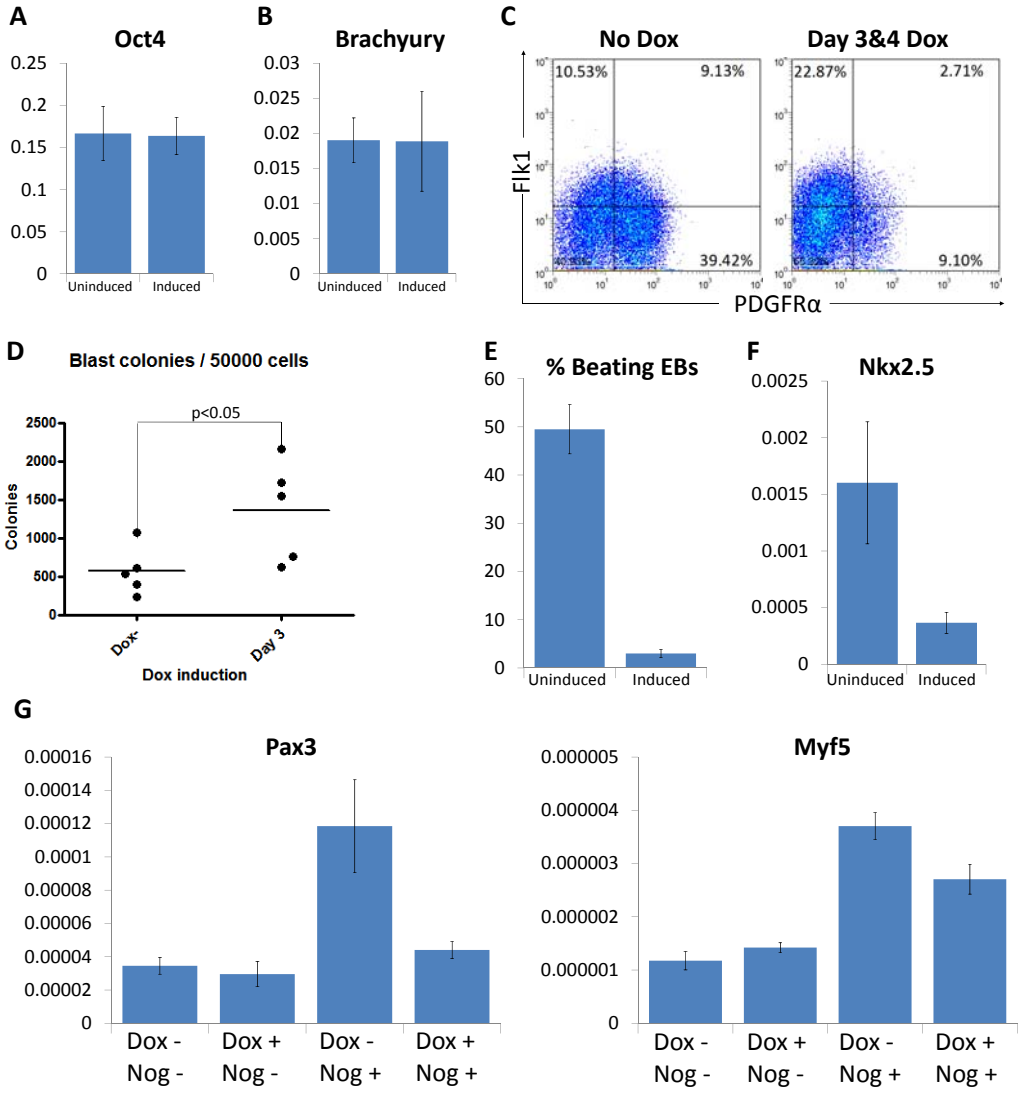


Figure 3.5: Effect of SCL on mesoderm lineages. qRT-PCR analysis of **A)** Oct4 and **B)** Brachyury expression. **C)** FACS analysis of Flk1 and PDGFRα surface markers on day 4. **D)** Summary of BL-CFC obtained from uninduced or induced day 3 iSCL EBs in repeated experiments. **E)** Functional analysis of cardiac output in the EBs through counting of beating foci. Percentage of the EBs beating in two independent experiments given. **F)** Real time PCR analysis for expression of cardiac marker Nkx2.5. Real time PCR analysis for expression of skeletal muscle markers. **G)**Pax3 and Myf5 with or without SCL induction, also with or without 30ng/ml Noggin treatment. Values shown are fold of GAPDH expression. The error bars represent standard error from three independent biological replicates.

meaningful conclusion. Since lateral plate mesoderm is formed in response to high levels of BMP4, while paraxial mesoderm requires lower levels, I treated the EBs with an inhibitor of BMP4, Noggin. This treatment increased the expression of the skeletal muscle specific transcription factors Pax3 and Myf5. Induction of SCL under these conditions decreased skeletal muscle differentiation (Fig 3.5G).

From these I concluded that SCL patterns mesoderm towards the hematopoietic lineage in expense of other lineages.

e. SCL acts downstream of BMP4

BMP4 is a patterning factor important in the development of lateral plate mesoderm (Maeno et al., 1996) and is reported to induce SCL expression (Mead et al., 1998). Based on my observations with Noggin-treated EBs, summarized in the previous section, I hypothesized that SCL can reverse the negative effect of BMP4 inhibition on hematopoiesis.

30 ng/ml of Noggin was added to the differentiating E14 or iSCL EBs after day 2 of differentiation for 2 days. This resulted in a decrease in the lateral plate mesoderm population on day 4 (Fig 3.6A). Confirming this decrease, the treatment also reduced the $CD45^+$ population in day 6 EBs (Fig 3.6B). Both these phenotypes were reversed when SCL was ectopically expressed simultaneously with the Noggin treatment (Fig 3.6A-B).

Based on the FACS data I have concluded that SCL is able to reverse the effect of the BMP4 inhibitor Noggin.

f. SCL acts cell autonomously

I hypothesized that SCL might be functioning by changing the levels of a key signaling molecule, e.g. BMP4. If this were correct, induction of SCL in a group of cells

Figure 3.6

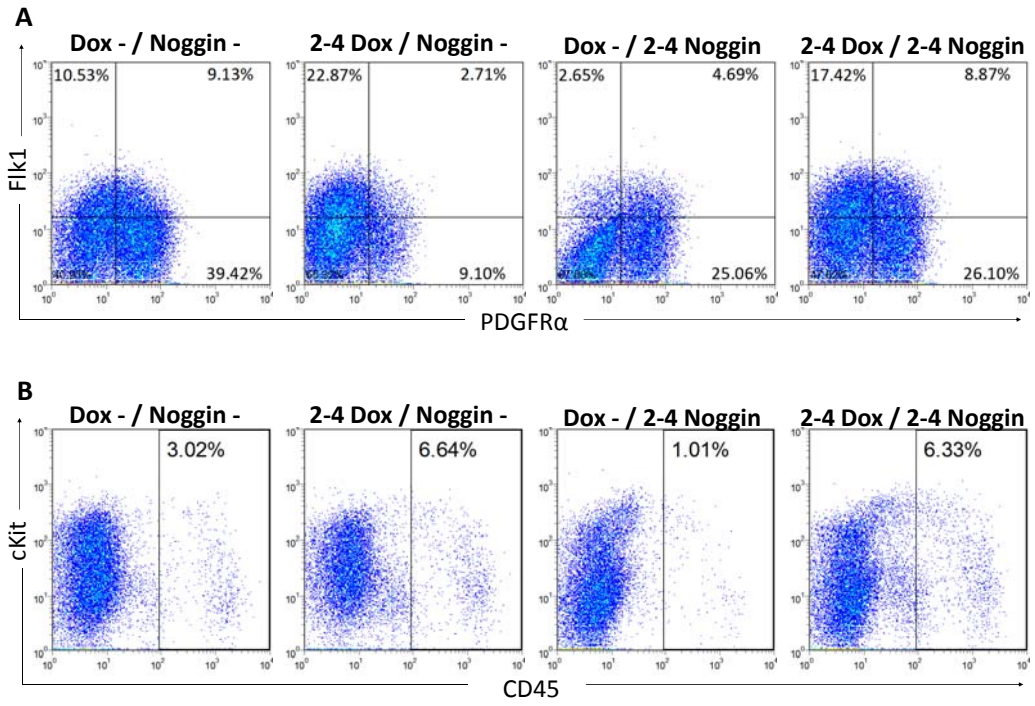
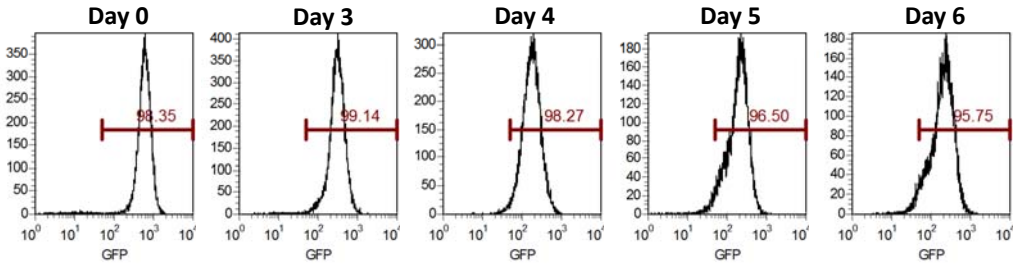


Figure 3.6: FACS data from representative experiments with 30 ng/ml Noggin treatment. **A)** Day 4 analysis of Flk1 and PDGFRα markers. **B)** Day 6 analysis of cKit and CD45 surface markers..

Figure 3.7

Clone #1



Clone #2

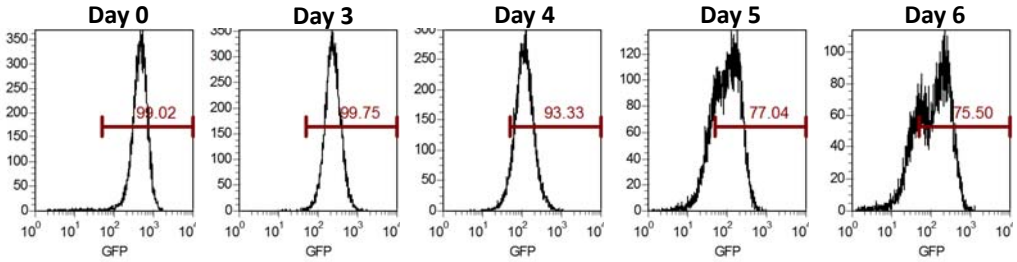


Figure 3.7: Analysis of two E14.GFP clones over a time course of differentiation. Clone #1 (top) keeps the same level of GFP expression, while clone #2 (bottom) silences. Clone #1 was used for further experiments.

Figure 3.8

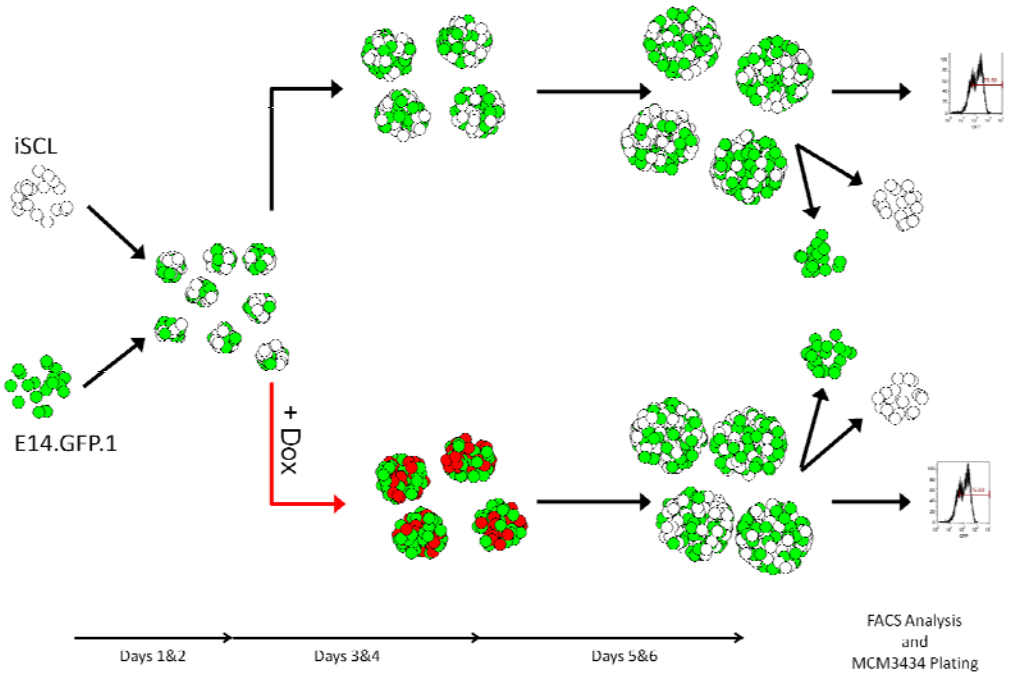


Figure 3.8: Schematic representation of cell autonomy experiment. Red indicates induced cells.

should have an effect on the uninduced cells in the same culture. In order to test this, I designed a cell-autonomy experiment in which EBs were formed from a mixture of iSCL cells and GFP-marked wild-type E14 cells and hematopoietic outcomes were analyzed separately.

A lentivirus expressing GFP under the control of the ubiquitin promoter was used to mark the E14 cells. After the infection, the cells were sorted, enriched and single cell cloned. 6 individual E14 clones were picked for further culturing. Their differentiation was followed over 6 days to eliminate clones that silenced GFP expression (Fig 3.7). Finally, clone #1 was selected to be used as the marked cell line (E14.GFP.1).

I mixed the iSCL and the E14.GFP.1 cells at a 1:1 ratio and differentiated as hanging drop EBs (Fig 3.8). One group of EBs was induced with Dox on days 3 and 4. On day 6 the levels of hematopoietic surface markers were analyzed and sorted GFP^- (iSCL) and GFP^+ (E14.GFP.1) cells were plated in semisolid medium for colony forming assay.

The percentage of the CD41 or CD45 expressing cells only increased in the GFP^- (iSCL) group (Fig 3.9A-B). Also, the number of colonies obtained from the GFP^- (iSCL) cells increased in response to induction, while the colony numbers from GFP^+ (E14.GFP.1) cells remained the same or slightly decreased (Fig 3.9C).

These data point to a cell autonomous function for SCL, disproving a mechanism based on a change in the level of a secreted signaling molecule to explain the mesoderm patterning effect of SCL.

Figure 3.9

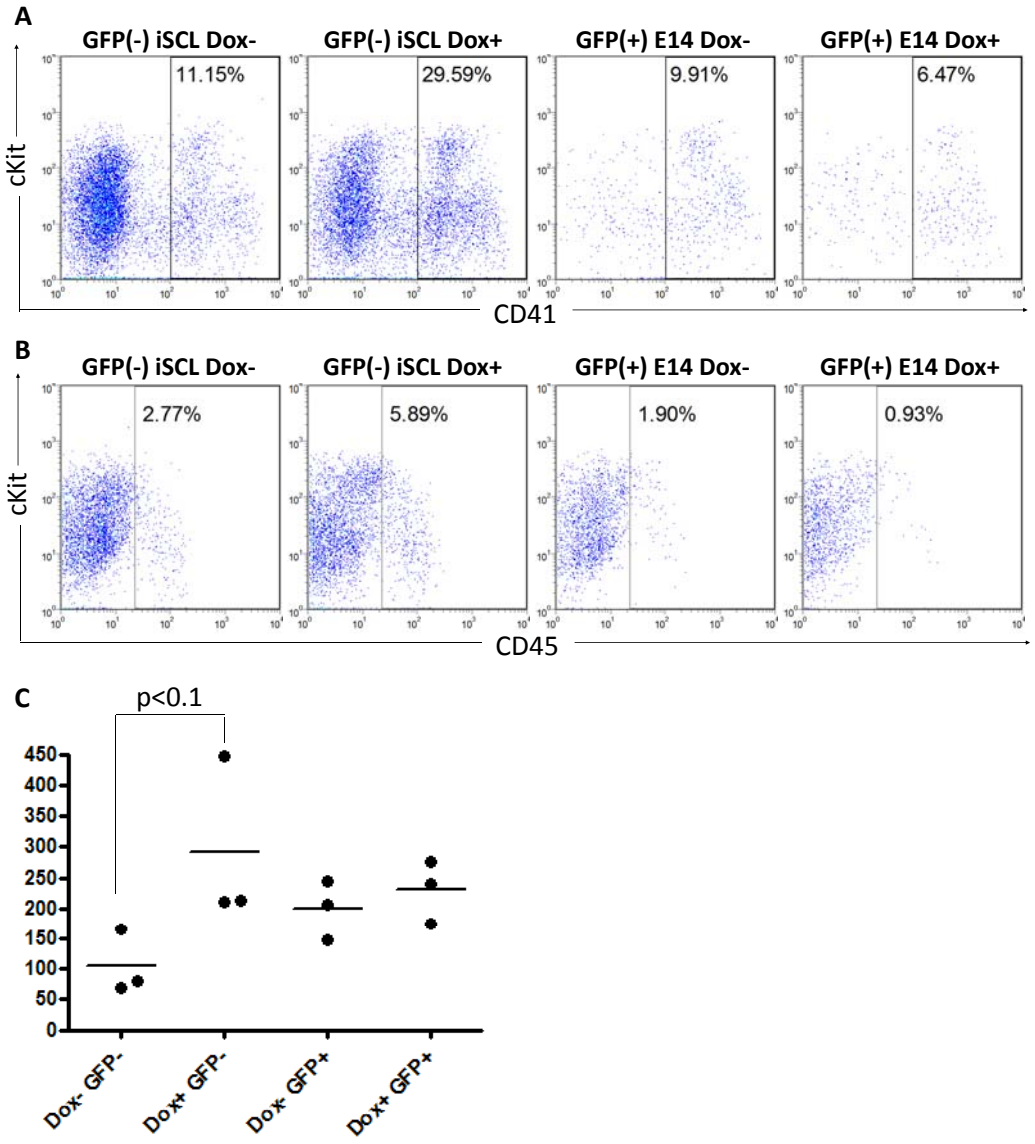


Figure 3.9 Results of cell autonomy experiments. FACS data from a representative experiment for CD41(A) and CD45(B) expression are shown. Two graphs on the left show iSCL cells from uninduced and induced samples, two on the right show E14.GFP.1. C) Summary of colony numbers in MCM3434 from three repeated experiments. Data was analyzed using the Mann-Whitney test.

g. Differentiation of SCL -/- cells

In order to see the effect of the loss of SCL on the EB differentiation, I compared the SCL-/- ES cells to the parental J1 ES cells. The expression of Brachyury was significantly higher in the SCL-/- cells. Also, the expression of the distal mesoderm genes Gsc, Chordin and FoxA2 were increased (Fig 3.10A). Still, the FACS profile of the cells looked similar on day 4 (Fig 3.10B). Thus, the increase in the level of Brachyury might signify a delay in mesoderm patterning, rather than production of more unpatterned mesoderm.

As expected, SCL-/- EBs were completely devoid of globin expression. The expression of cardiac marker Nkx2.5 was increased. The absence of the hematopoietic lineage might have allowed more unpatterned mesoderm cells to differentiate towards cardiac lineage (Fig 3.10C).

h. VP16-SCL fusion protein induces hematopoietic cell proliferation in culture

SCL has been reported to function both as an activator (Krosl et al., 1998) and a repressor (Vitelli et al., 2000). I hypothesized that one of these roles would dominate during early development. In order to test this, cell lines were created to express the VP16-SCL and Eng-SCL fusion proteins inducibly, which is a technique used to convert a transcription factor to an obligate activator or a repressor respectively (Steiner et al., 2006; Wang et al., 2006).

The activating and repressing fusions of SCL acted similar to SCL in the mesoderm patterning assay (not shown). However, I observed a differentiation block caused by the late ectopic expression of VP16-SCL (data not shown) and hypothesized that this protein is keeping the cells in an undifferentiated state.

Figure 3.10

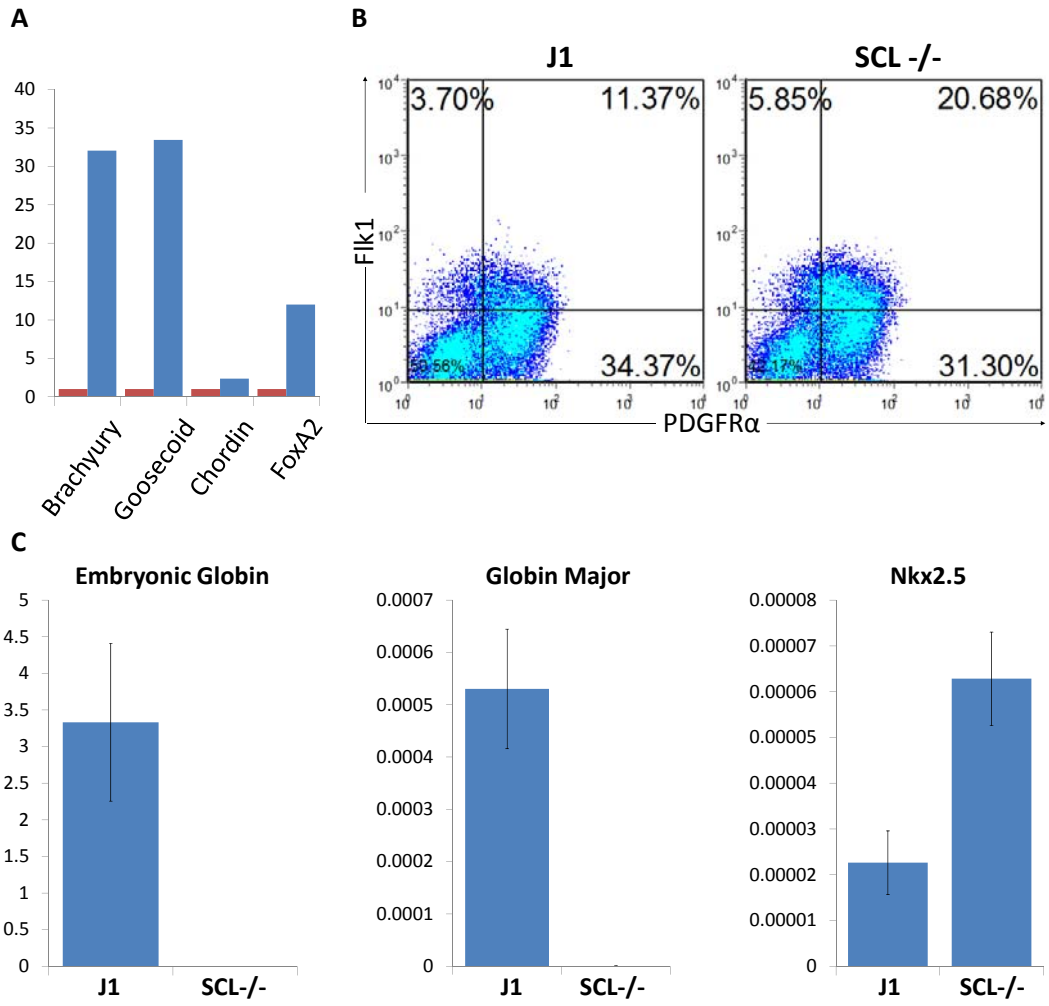


Figure 3.10: Differentiation of SCL-/- EBs. **A)** Fold change in the expression of mesodermal genes between J1 and SCL-/- cells. Values shown are normalized to the expression level in the J1 EBs. **B)** FACS analysis of the EBs on Day 4. **C)** qRT-PCR analysis of hematopoietic and cardiac markers on day 6 EBs. The error bars represent standard error from three independent biological replicates.

Co-culture with the OP9 stromal cell line is commonly used to support the growth of the hematopoietic stem cells in culture (Nakano et al., 1994). However, even in the presence of cytokines and on an OP9 feeder layer, hematopoietic progenitor or stem cells will lose their multipotency after a few days, stop proliferating and differentiate into terminal hematopoietic cell types. Ectopic expression of a small number of genes, including HoxB4 (Kyba et al., 2002) and the oncogene Bcr-Abl (Perlingeiro et al., 2001) have been shown to promote self-renewal of HSCs under these conditions.

In order to test whether VP16-SCL can support the proliferation of hematopoietic progenitors, $cKit^+/CD41^+$ cells were sorted from the uninduced day 5 iVP16SCL EBs and plated on an OP9 feeder layer with or without Dox induction. Cells in the induced wells began proliferating strongly, while the number of cells in the uninduced wells declined after an initial burst of growth. The induced cells continued proliferating until the end point of the experiment, day 38 (Fig 3.11A). The growing cells were homogeneous in terms of morphology and surface marker phenotype. The small, round morphology and $cKit^+/CD41^+/CD45^+$ surface phenotype (Fig 3.11 C, D) suggested an undifferentiated, progenitor cell type. This was also supported by the blast-like appearance of the cells in cytospin preparations (Fig 3.11B, right panel) and formation of the mixed lineage colonies in CFC assays.

Removal of the ectopic protein induction was expected to stop proliferation and allow differentiation in culture. However, iVP16SCL cells continued proliferating more than 10 days after the Dox removal. Even though the cells in cytospins preparations were heterogeneous in morphology (Fig 3.11B, left panel), differentiation was inefficient in

Figure 3.11

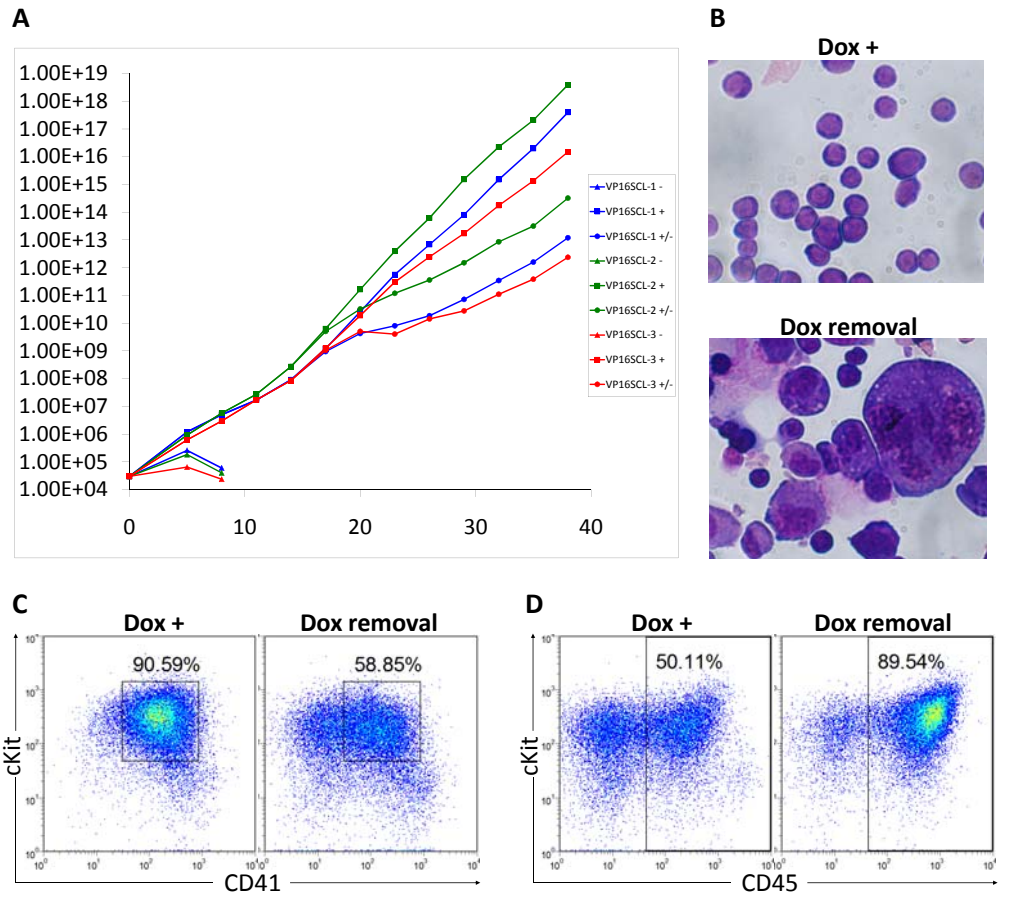


Figure 3.11: The growth of iVP16-SCL cells on OP9. **A)** The growth curve for three independent iVP16-SCL clones w/o Dox, with Dox and after Dox removal. **B)** Cytospins from and **C-D)** Surface profiles of cultures that are continuously induced or cultured in media w/o Dox after 17 days of induction.

general. This shows that the cells have gained the ability to continue proliferation without an external factor.

Discussions and Conclusions

Using the EB differentiation model, I have mapped the window of SCL function during early development to the mesoderm patterning stage. The effect of ectopic SCL expression is highest on day 4 of differentiation. This expression window corresponds to the highest Brachyury expression in the EB. Also, $Flk1^{+}/PDGFR\alpha^{-}$ or $PDGFR\alpha^{-}/Flk1^{+}$, which correspond to the patterned mesoderm lineages, are not yet seen. The culture is dominated by $Flk1^{+}/PDGFR\alpha^{+}$ cells. From these we conclude that the beginning of day 4 in our culture system is when mesoderm initiation peaks and unpatterned mesoderm is at the highest level. The effect of SCL on day 3 is smaller, because mesoderm is still being formed. On day 5, most of the mesodermal cells available are committed to a lineage and thus are not affected by SCL expression.

The data obtained from the *Xenopus* and zebrafish experiments on mesoderm patterning in response to SCL expression are contradictory. In *Xenopus* experiments SCL overexpression did not affect the dorsal-ventral patterning of the embryo (Mead et al., 1998). However, ectopic expression of SCL in zebrafish increased hematopoietic and endothelial tissue at the expense of pronephric and somatic mesoderm (Gering et al., 1998). Our findings are thus similar to the zebrafish data. *Xenopus* embryos were ventralized only when SCL was co-expressed with LMO2 (Mead et al., 2001). This suggests that differences between model systems might be due to differences in levels of LMO2 expression.

In another study where SCL was ectopically expressed, no change in PDGFRα expression was observed (Endoh et al., 2002). However, in this study, the cells were differentiated on OP9 cells, which are known to skew differentiation in favor of hematopoiesis and thus might produce only minimal amounts of paraxial mesoderm (Nakano et al., 1994).

Although I observed an increase in hemangioblast formation in response to SCL expression, I did not observe a significant and reliable increase in the endothelial lineage. The bi-lineage (lateral plate and paraxial mesoderm) origin of endothelial cells complicates the analysis (Pardanaud et al., 1996). SCL might be increasing the endothelial cells of the lateral plate origin, while decreasing those produced from the paraxial mesoderm. An increase in endothelial differentiation was also not observed when SCL was transfected into the ES cells differentiating on OP9 (Endoh et al., 2002).

One possible explanation for the observed decrease in the paraxial mesoderm and increase in the lateral plate mesoderm in response to SCL expression is increased proliferation of lateral plate mesoderm cells. However, if this were the case, I would have seen an increased number of GFP^- iSCL cells in the induced chimeric EBs. The fact that the ratio of GFP^- cells to GFP^+ cells in the chimeric EBs was not affected by SCL expression shows that SCL does not change the proliferation dynamics significantly, but rather affects the fate of the cells.

BMP4 is one of the main signaling factors involved in the initiation of hematopoiesis (Maeno et al., 1996) and it induces the SCL expression (Mead et al., 1998). Therefore, I hypothesized that ectopic SCL expression could rescue inhibition of BMP4. Ectopic SCL expression recovered hematopoiesis in response to inhibition of

BMP4 with Noggin treatment during mesoderm patterning. The levels of hematopoiesis in response to SCL in cultures treated or not treated with Noggin were comparable. This shows that SCL does not rescue the loss of BMP4 signaling phenotype by simply increasing the level of BMP4 expression.

Unexpectedly, the colony numbers in methylcellulose were not in line with the FACS data in these experiments (not shown). Even though Noggin-treated EBs had significantly less hematopoietic progenitors by FACS analysis, CFC numbers remained at similar levels. Sakurai and colleagues reported that $Flk1^+$ lateral plate and $PDGFR\alpha^+$ paraxial mesoderm cells can switch fates during early differentiation (Sakurai et al., 2006b). I hypothesized that cells in our cultures would switch their fate after removal of Noggin. In order to test this hypothesis, I extended the Noggin treatment until day 6 of differentiation in some samples. In E14 EBs this resulted in decreased colony numbers, but iSCL EBs still did not respond as expected. The methylcellulose assay requires an additional 6 days of culture after the EBs and Noggin treatment, which may still be enough for non-hematopoietic cells to switch to a hematopoietic fate, especially in a culture environment favoring hematopoietic cells.

Induction of an extracellular signaling molecule by SCL is another plausible mechanism that can explain my findings. I tested this hypothesis by mixing wild type and SCL inducible cells in the same EBs. An extracellular signal released in response to the SCL expression would be expected to increase hematopoiesis also in wild type cells. However, the hematopoietic output did not change in wild type cells after Dox treatment. Therefore, I concluded that the SCL function is cell autonomous.

The most significant difference between wild-type and SCL-/- cells was the increase in the levels of distal primitive streak genes FoxA2 and Chordin. This suggests a role for SCL in promoting the proximal primitive streak fate. These findings will be discussed further in the next chapter.

It is worth noting that SCL, a protein involved in erythropoiesis (Green et al., 1991) does not increase the colony forming activity when expressed on days 5 and 6, a time window where early erythrocyte progenitors are available. Based on the results of studies performed in hematopoietic cell lines (32D, a late myeloid progenitor cell line and HL-60, a leukemic cell line), SCL has been reported to increase proliferation and block differentiation (Condorelli et al., 1997). The absence of this effect in EB-based progenitors suggests that it is not universal and progenitors in the day 5 and 6 EBs do not respond to SCL expression.

SCL is an oncogene and it has been shown to increase proliferation of the hematopoietic cells (Condorelli et al., 1997). Still, its ectopic expression neither blocks the differentiation nor helps the proliferation of ES derived progenitors on OP9 (data not shown). However, I observed that a fusion protein, VP16-SCL can initiate the proliferation of hematopoietic progenitors. These data suggest that the observed VP16SCL phenotype is the result of switching SCL from a transcriptional repressor to an activator. Wild type SCL might silence certain self renewal associated genes, thus allowing the differentiation to proceed.

The lack of a slow growth or plateau phase, during which a fast dividing clone could overtake the culture, suggests that the transformation of the cells in response to VP16-SCL expression is not a rare event (e.g. mutation). A likely explanation is the

initiation of a self-propagating stable state is keeping the cells in an undifferentiated state. This model may be useful to study self-renewal of hematopoietic progenitors and to identify factors that might induce their proliferation in culture.

CHAPTER 4: PROBING MOLECULAR INTERACTIONS OF SCL DURING MESODERM PATTERNING

Introduction

SCL has been shown to interact with a number of proteins. It cannot form homodimers, but can heterodimerize with E12 or E47 proteins to bind DNA (Hsu et al., 1991). This binding determines the preferred DNA binding sequence for SCL (Hsu et al., 1994). However, a DNA binding mutant of SCL in which three conserved residues (RER) shown to be required for DNA binding in other bHLH proteins were mutated to Alanine (SCL-RERAAA), was able to rescue hematopoiesis when expressed in SCL-/- cells (Porcher et al., 1999). On the other hand, when a heterodimerization mutant of SCL (F207A-L210A) was tested for the rescue hematopoiesis was absent. This shows that even though the DNA binding is dispensable, interaction with the E2A proteins was still necessary for the SCL function. The researchers did observe a decreased number of erythrocytes and megakaryocytes in SCL-RERAAA rescued EBs and concluded that even though the DNA binding is dispensable at an early stage, it was required for lineage specific actions of SCL. Therefore it is plausible that different interactions occur at different stages.

SCL complex

SCL also associates closely with LMO2 (Valge-Archer et al., 1994; Wadman et al., 1994). Although this interaction was discovered in erythroleukemia (MEL) and T cell leukemia (Jurkat) cell lines, the requirement for LMO2 in early hematopoiesis suggests that SCL and LMO2 work together in early development (Yamada et al., 1998). A

mutational analysis of the SCL HLH region showed a phenylalanine residue on the second helix (F238) to be critical for the hematopoietic function (Schlaeger et al., 2004). Mutation of this residue to a Glycine (SCL-F238G) decreased the amount of LMO2 bound to SCL. LMO2 binding was also disrupted in SCL mutants that cannot form heterodimers with E2A proteins. However, findings summarized in this publication and observations of another group (Wadman et al., 1994) show that E2A proteins and LMO2 do not directly interact in two hybrid experiments. Therefore, it is possible that some of the residues mutated in heterodimer-deficient SCL variants might also be required for the LMO2 binding. A more recent mutational analysis discovered two more helix residues involved in LMO2 binding and also showed that the SCL binding prevents proteosomal degradation of LMO2 (Lecuyer et al., 2007).

Through LMO2, SCL interacts with GATA1 and GATA2 proteins (Osada et al., 1995). GATA1 is required for erythropoiesis (Pevny et al., 1991). GATA1 deficient cells injected into the wild type blastocysts contributed to all tissues, including blood, except for erythrocytes. Lack of GATA2, on the other hand, leads to defects in progenitors higher in the hematopoietic hierarchy (Tsai et al., 1994). Although GATA2 knockout cells contributed to primitive erythropoiesis early on in the chimeric animals, they disappeared from the blood lineage by the time definitive hematopoiesis started. When erythroid progenitors are induced to terminally differentiate, GATA2 expression decreases, while GATA1 expression stays steady or increases (Leonard et al., 1993). Finally, overexpression of GATA2 in erythroid progenitor cells blocks differentiation and increases proliferation (Briegel et al., 1993). Based on these data, it has been suggested that GATA2 is initially active in the erythroid progenitors, but as the cells mature it is

replaced by GATA1. In fact, GATA2 expression is significantly higher in erythroid cells devoid of GATA1, suggesting a direct transcriptional regulation (Weiss et al., 1994).

There is contradictory data regarding the involvement of GATA2 in self-renewal of non-erythroid blood progenitors. Experiments with an inducible form of GATA2 showed that the overexpression of the protein resulted in the differentiation of non-erythroid progenitor cell lines (Heyworth et al., 1999). Overexpression of GATA2 also induces quiescence in HSCs (Tipping et al., 2009). However, in embryos lacking only one copy of GATA2, HSC proliferation was hampered (Ling et al., 2004). Phenotypes of GATA2 (Tsai et al., 1994) and Evi1 (Yuasa et al., 2005) knockout animals, which have low levels of GATA2, and data obtained from *in vitro* differentiation of GATA2-/- ES cells (Tsai and Orkin, 1997) imply that cells are sensitive to the level of GATA2 expression. A certain level is necessary for self-renewal and proliferation, while high level expression causes initiation of differentiation pathway.

SCL has been shown to interact with the transcriptional repressor complex mSin3A and HDAC1 in MEL cells (Huang and Brandt, 2000). Surprisingly, SCL also has been shown to interact with the coactivator p300 complex in the same cell line (Huang et al., 1999). The SCL-p300 interaction becomes prominent when MEL cells are induced to differentiate. Under these conditions, the SCL-mSin3A interaction weakens, suggesting a change in the function of the complex from a repressor to an activator. Acetylation of SCL by p/CAF has been shown to regulate this switch by decreasing the strength of SCL-mSin3A binding (Huang et al., 2000).

In vitro experiments show that SCL can bind to both mSin3A (Huang and Brandt, 2000) and p300 (Huang et al., 1999) directly. Nevertheless, expression of SCL

by itself or with E47 and GATA2 is not sufficient to induce expression of reporter genes under the control of SCL target promoters (Lahlil et al., 2004; Lecuyer et al., 2002). This means that recruitment of factors by other SCL complex members is necessary either for transcriptional activation/repression or DNA binding.

Another key member of the SCL complex is LDB1 (Lahlil et al., 2004; Wadman et al., 1997). LDB1 is recruited to the complex through its interaction with LMO2 and is required for activation of globin expression by the SCL complex (Song et al., 2007). The role of LDB1 is thought to be linking of protein complexes far apart on the DNA through homodimerization (Xu et al., 2003).

ETO2 is a member of the SCL complex in erythrocyte progenitors (Goardon et al., 2006). In the complex it binds to E2A and represses targets of the complex. As the cells differentiate, ETO2 is removed from the complex and genes are activated. Knockdown of ETO2 results in premature activation of SCL target genes. Interestingly, an alternative pathway for SCL target gene repression/activation through direct interaction of SCL with mSin3A and p300, has been suggested (Huang and Brandt, 2000; Huang et al., 1999). Since loss of ETO2 switches the character of the complex, the mSin3A/p300 interaction might be secondary to the ETO2 interaction.

The SWI/SNF family member BRG1 is another member of the complex involved in the repression of the targets in erythrocyte progenitors (Xu et al., 2006). Like ETO2, BRG1 is removed from the complex as the cells mature. For transcriptional repression, BRG1 recruits mSin3A and HDAC2. Overexpression of BRG1 represses the transcription of a target gene, P4.2, but a knockdown has not been performed. Therefore it is unknown whether BRG1 is required for the maintenance of the repression. Also

unknown is which protein BRG1 binds to in the complex. BRG1 has been shown to interact with GATA4 in a different context (Lickert et al., 2004), but GATA1 does not co-immunoprecipitate with BRG1 (Xu et al., 2006).

Targets of SCL

Based on the data on the structure of the SCL complex, the search for transcriptional targets of SCL concentrated on the genes with an E-box and a GATA binding site placed in close proximity on their promoters (Wadman et al., 1997). GATA1 in erythrocytes and megakaryocytes (Vyas et al., 1999), P4.2 (Xu et al., 2003) and Glycophorin A (Lahlil et al., 2004) in erythrocytes are target genes that fit this profile. E-box and GATA motifs were found to be the most common binding sequences for SCL in a recent high-throughput study, where SCL-bound sites in a hematopoietic progenitor cell line were analyzed using the ChIP-Seq method, supporting the E-box/GATA model of SCL complex binding (Wilson et al., 2009).

In vascular endothelial cells the expression of Flk1 is induced by the SCL complex and is dependent on the E-box and GATA binding sites (Kappel et al., 2000). However, these sites are not adjacent to each other. Also, only the mutation of 1 of 3 GATA binding sites completely abolishes Flk1 promoter controlled reporter gene expression in transgenic animals. Some transgenic animals carrying constructs mutated at either one of the 2 remaining GATA sites or the 2 SCL sites still express Flk1 in an endothelial-specific manner. SCL was shown to bind the promoter region, but further testing with reporter constructs that contain multiple mutations is required to reveal a complete picture.

The E-box/GATA consensus binding sequence for the SCL complex seems to be limited to the erythroid lineage. One significant difference between erythroid and non-erythroid functions of SCL is the requirement for the DNA binding domain. The DNA binding function of SCL is dispensable for early hematopoietic development (Porcher et al., 1999), while it is required for the erythroid maturation (Kassouf et al., 2008) and P4.2 expression (Xu et al., 2003). Therefore, the E-box binding sequence might be irrelevant for the mesoderm pattering stage SCL targets (Wadman et al., 1997). In fact, in the gene regulatory network made up of SCL, GATA2 and Fli1; E-boxes, presumptive binding sites for SCL, are less important than the GATA and Ets sequences (Pimanda et al., 2007). Finally, the presence of the GATA factors in the SCL complex during mesoderm patterning has not been confirmed and therefore a GATA site may also not exist on the promoters of the gastrulation stage SCL targets.

c-Kit was identified as an SCL target in TF-1 cells (Krosl et al., 1998), which have hematopoietic progenitor characteristics (Kitamura et al., 1989). Unlike erythroid cell line targets, the portion of the c-Kit promoter required for the activation by the SCL complex does not have adjacent E-box and GATA binding sites (Lecuyer et al., 2002). Instead, the complex binds the promoter through a conserved Sp1 binding site. *In vitro*, Sp1 interacts with SCL, LMO2, LDB1 and GATA1; but not with E47. How Sp1 interacts with the complex *in vivo* is currently unknown.

Runx1 and Runx3 were shown to be upregulated after the reintroduction of SCL to an SCL-/- yolk sac cell line (Landry et al., 2008). SCL and complex members LMO2 and GATA2 were shown to bind the Runx promoters in fetal liver cells. However, the E-boxes and GATA sites on the promoters are not placed closely on the promoter as would

have been predicted from the structure of the SCL complex. Therefore, SCL and GATA2 might be acting separately on the same promoter. In this study, the presence of evolutionarily conserved E-boxes was one of the criteria in selecting candidate target genes, therefore targets where SCL does not bind to an E-box sequence, but acts through another DNA binding factor were eliminated from the dataset.

Almost all of the work on the SCL complex to date was performed in hematopoietic cell lines. Having established "when" SCL functions in the EB model, I began using EB differentiation to gain insight on the mechanism of SCL action. I applied an inducible expression strategy to evaluate the activity of two key SCL complex members, LMO2 and GATA2 and two SCL mutants, LMO2-binding defective SCLF238G and DNA-binding defective SCL-RERAAA. A cell line inducible for the expression of an *in vivo* biotinylated version of SCL was also created. Using this cell line I performed IP experiments on mesoderm patterning stage EBs to co-purify proteins SCL interacts with at this stage of development. To the best of my knowledge, no targets of SCL during early development have been identified so far. I performed transcriptional profiling after 6-hr induction of SCL to identify transcriptional targets of SCL.

Results

a. Ectopic expression of SCL complex members LMO2 and GATA2

I hypothesized that, if LMO2 and GATA2 are in a complex with SCL during early development, their ectopic expression would have a similar phenotype to that of SCL, by promoting the formation of the SCL complex. To test this, iLMO2 and iGATA2

cell lines were created and time course experiments similar to the ones in the previous chapter were performed.

As with SCL, I first analyzed the expression of LMO2 and GATA2 mRNAs in wild-type ES cells. Both genes are expressed after day 3 and peak around day 4 like SCL. (Fig 4.1).

Ectopic expression of LMO2 only on day 4 led to an increase in the amount of $CD41^+$ and $CD45^+$ cells, albeit smaller than in the case of SCL (Fig 4.2A-D). The increase in the number of hematopoietic colonies from this sample was also statistically significant. Unlike SCL, however, continuous expression of LMO2 from day 3 to day 6 of differentiation did not promote hematopoiesis (Fig 4.2E).

This limited positive effect window suggests that LMO2 and SCL are working together at one stage of differentiation, day 4 in the EBs. At earlier or later time windows this may not be the case.

Expression of GATA2 at early time points (day3 and 4) did not increase hematopoietic output. In fact, day 4 expression slightly decreased the number of $CD45^+$ cells (Fig 4.3A-D). Surprisingly, ectopic GATA2 expression on days 5 and 6, while strongly reducing the $CD45^+$ mature hematopoietic population, increased the percentage of the immature $CD41^+$ cells (Fig 4.3E). This apparent delay in differentiation was reflected in colony forming assays. Plates containing cells from samples induced on day 5 and day 6 had more colonies in general. Also, the number of large, mixed lineage colonies, descending from immature, highly proliferating progenitors, was higher in these cultures.

Figure 4.1

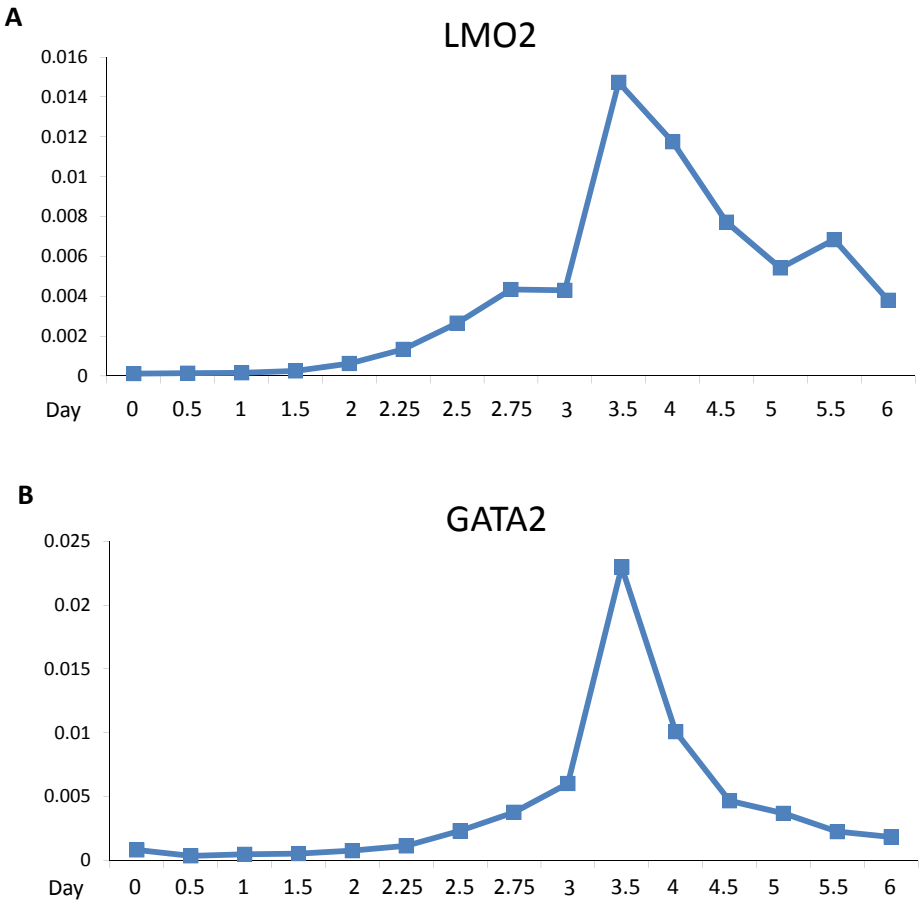


Figure 4.1: Expression of LMO2 (**A**) and GATA2 (**B**) over a time course during EB differentiation of E14 cells.

Figure 4.2

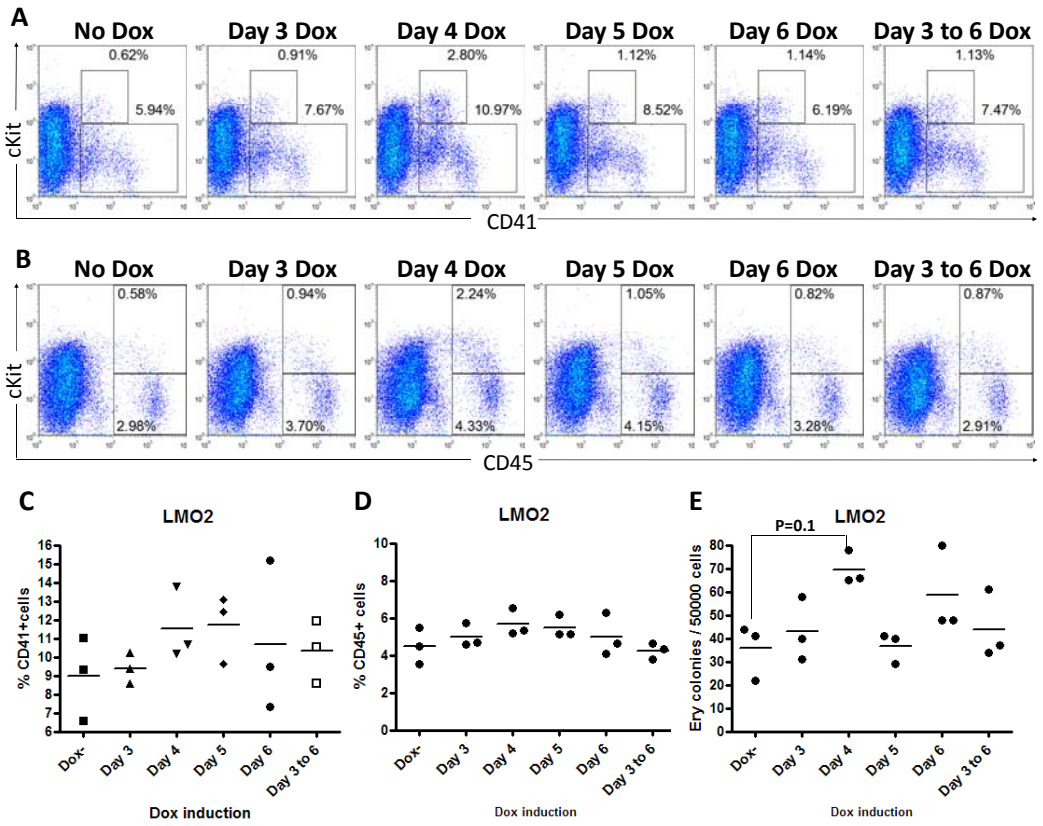


Figure 4.2: Data from LMO2 24 hr time course experiments. Surface phenotype of differentiated iLMO2 EBs with cKit/CD41(**A**) and cKit/CD45(**B**) stainings from a representative experiment. Percentage of $CD41^{+}$(**C**) and $CD45^{+}$(**D**) obtained at repeated experiments. **E)** Number of colonies obtained at repeated experiments. Data analyzed using Mann-Whitney test

Figure 4.3

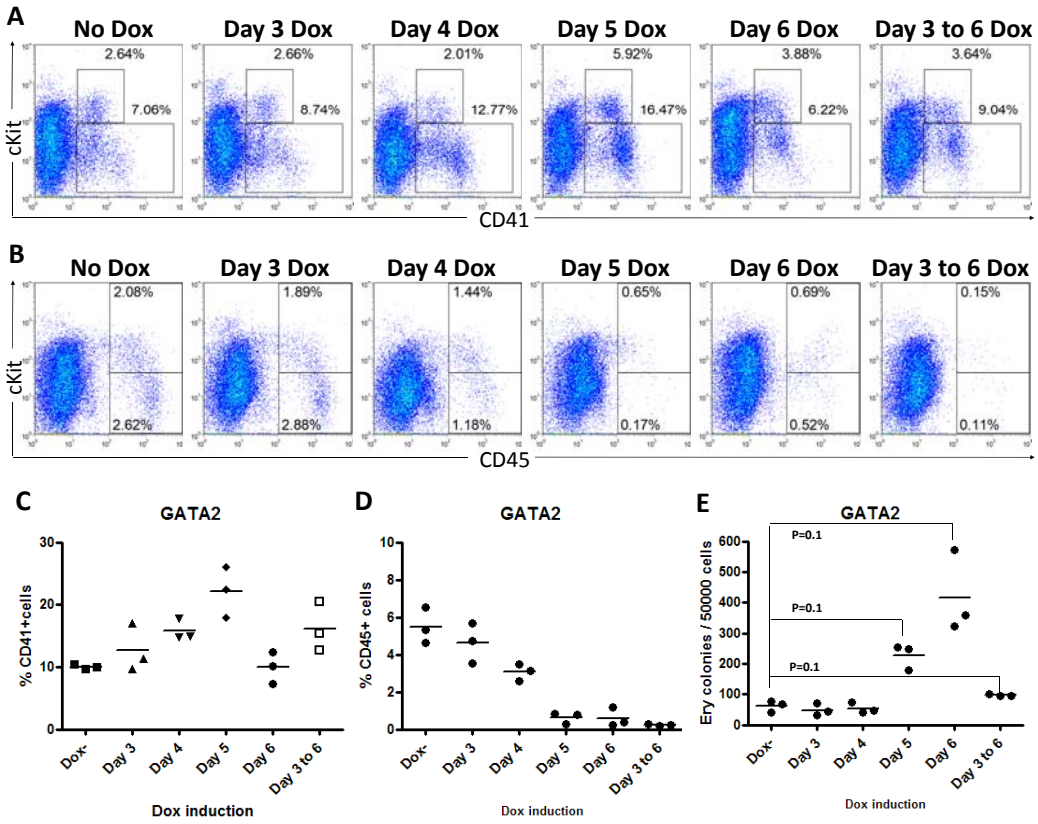


Figure 4.3: Data from GATA2 24 hr time course experiments. Surface phenotype of differentiated iGATA2 EBs with cKit/CD41(**A**) and cKit/CD45(**B**) stainings from a representative experiment. Percentage of $CD41^{+}$(**C**) and $CD45^{+}$(**D**) obtained at repeated experiments. **E)** Number of colonies obtained at repeated experiments. Data analyzed using Mann-Whitney test

This late stage phenotype of GATA2 expression is in line with earlier reports about the involvement of the protein in the proliferation and self renewal of hematopoietic progenitors (Tsai and Orkin, 1997). However, expression of GATA2, even though it blocked differentiation, was not enough to keep ES-derived hematopoietic progenitors growing on OP9 feeders (not shown).

To see if these two transcription factors have a mesoderm programming function, I analyzed the EBs on day 4 of differentiation, after 2 days of induction (Fig 4.4A-B). Neither LMO2 nor GATA2 had SCL-like mesoderm patterning activity. Ectopic expression did not affect the amount of presumptive lateral plate or paraxial mesoderm progenitors. Supporting this finding, induced iLMO2 and iGATA2 EBs also did not have increased numbers of BL-CFC (Fig 4.4C-D).

b. Ectopic expression of SCL mutants

I decided to take a genetic approach as an alternative way of studying SCL complex function during early development and created cell lines expressing two known mutants of SCL, LMO2-binding defective SCLF238G and DNA-binding defective SCLRERAAA (Schlaeger et al., 2004). Earlier work with SCL-/- ES cells (Schlaeger et al., 2004) and zebrafish embryos treated with SCL morpholinos (Patterson et al., 2007) determined that the DNA binding mutant (SCLRERAAA) is sufficient to rescue the loss of hematopoiesis, but LMO2 binding mutant (SCLF238G) is not. However, the readout in these experiments was the emergence of the erythrocytes, which requires the mutant to rescue the SCL function in multiple steps of the development (mesoderm patterning, lateral plate differentiation and hematopoietic progenitor differentiation). On the other

Figure 4.4

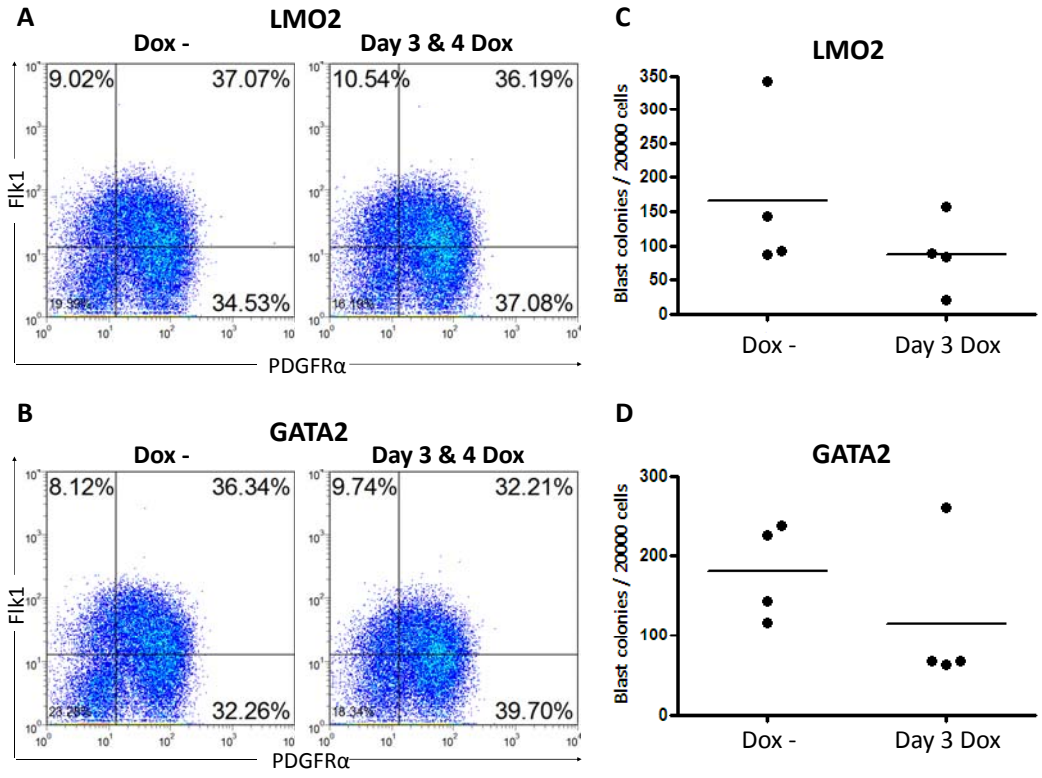


Figure 4.4: Effect of ectopic LMO2 (**A**) and GATA2 (**B**) expression on mesoderm markers Flk1 and PDGFRα on day 4 of differentiation. Number of blast colonies obtained from uninduced or induced iLMO2(**C**) and iGATA2(**D**) EBs on day 3.

hand, timed ectopic expression of mutants in differentiating ES cells allows us to analyze the function at different time points separately.

Expression of both SCLF238G and SCLRERAAA during the mesoderm patterning stage increased the presumptive lateral plate progenitor population on day 4 of EB differentiation. However, a significant decrease in other mesodermal progenitors ($PDGFR\alpha^{+}$) was only seen in the iSCLRERAAA cells, not in the iSCLF238G cells (Fig 4.5).

The increase in the hematopoietic compartment on day 6 of differentiation was stronger in iSCLRERAAA cells. The effect of SCLF238G was not consistent (Fig 4.6).

The data support earlier findings and show that an interaction between SCL and LMO2 is required for SCL to pattern mesoderm towards hematopoietic fate, but that DNA binding at this stage might be dispensable.

c. Biochemical analysis of the SCL complex at the mesoderm patterning stage

I tested several SCL antibodies, but could not find any that was strong enough, or did not have too many non-specific targets, for the successful purification of a protein complex. Therefore, I decided to ectopically express a tagged version of SCL in differentiating ES cells. *In vivo* biotinylation, which has previously been used to tag SCL in MEL cells for a similar purpose, was employed (Goardon et al., 2006). The inducible cassette has a version of SCL tagged with an *in vivo* biotinylation sequence (Avi-tag) on its amino terminus (Tirat et al., 2006). Our tag is distinct from previously used tags in that it also incorporates a TEV protease cleavage site for specific elution under native conditions. Finally, the E.coli biotin ligase, BirA, was co-expressed from the same transcript with an IRES sequence (Fig 4.7A).

Figure 4.5

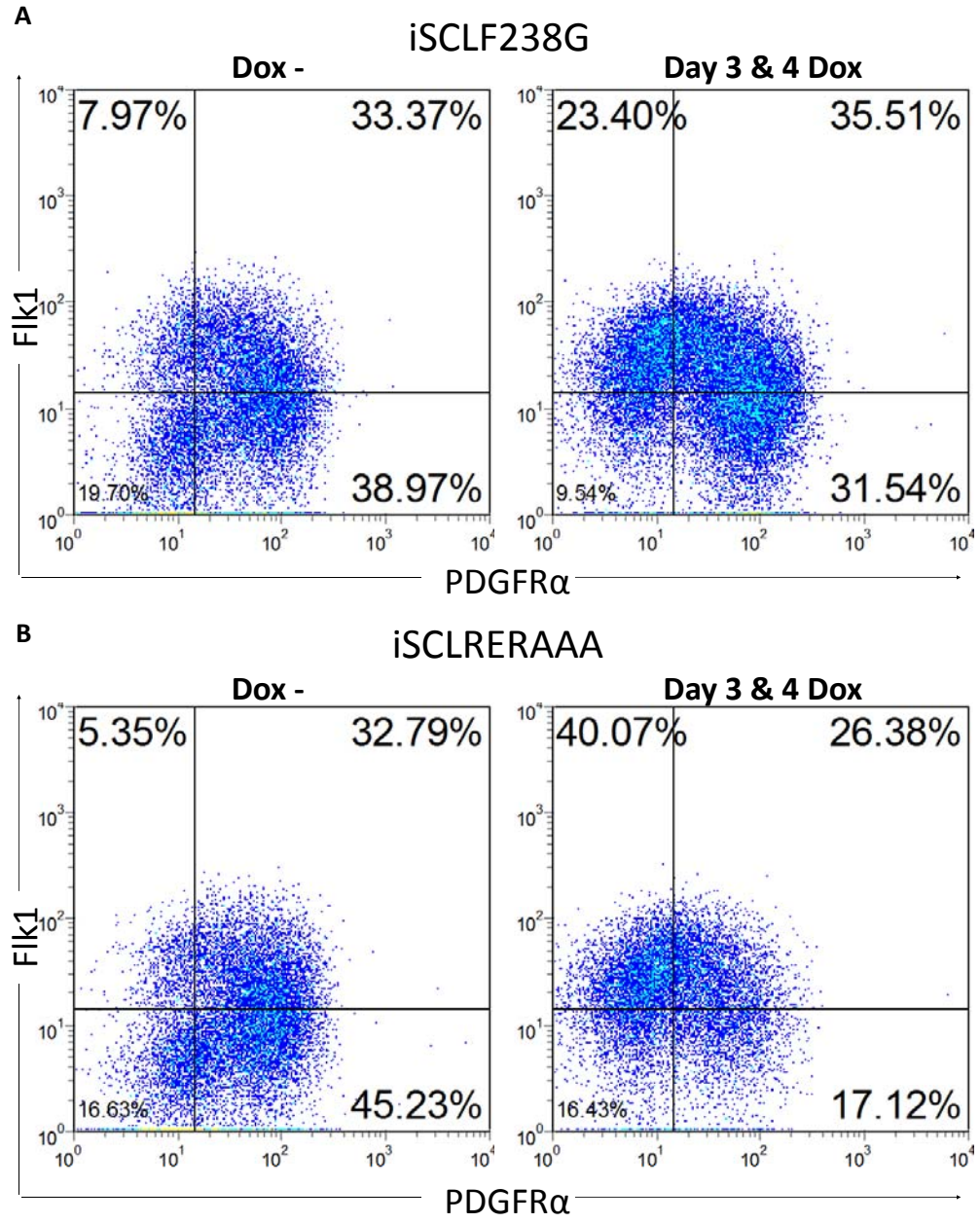


Figure 4.5: Surface phenotype of uninduced and induced day 4 iSCLF238G and iSCLRERAAA EBs in a representative experiment.

Figure 4.6

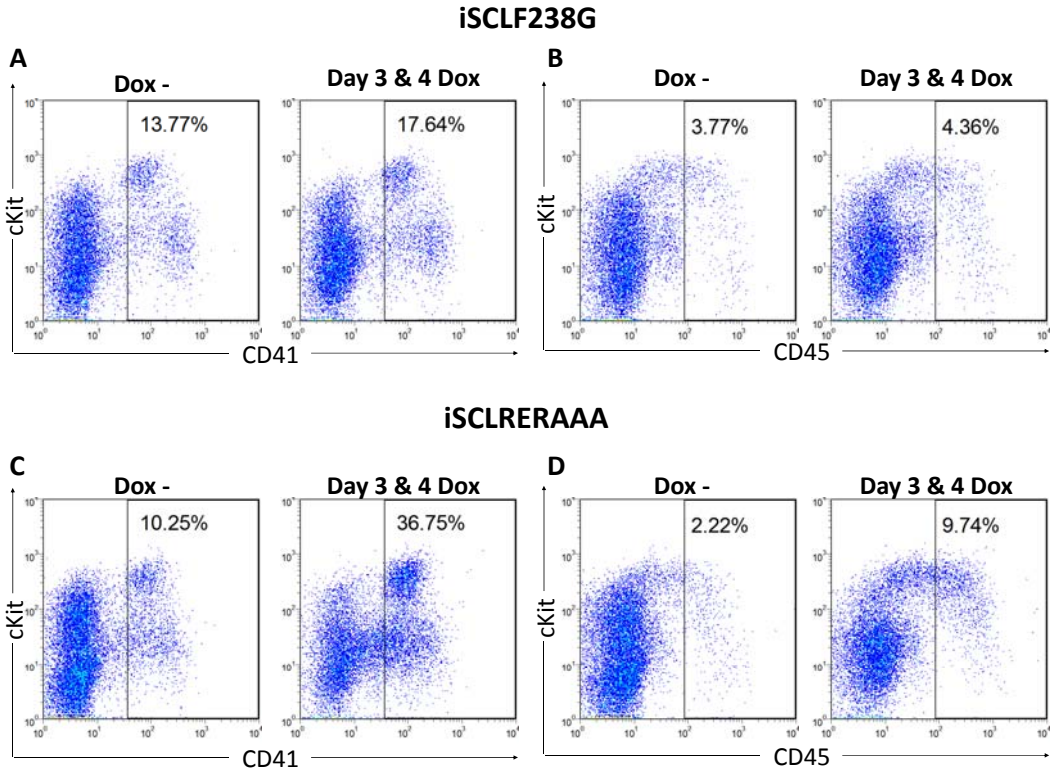


Figure 4.6: Expression of cKit/CD41 and cKit CD45 markers on day 6 iSCLF238G (**A-B**) and iSCLRERAAA (**C-D**) EBs.

Expression of SCL in the iAvi-SCL cell line was confirmed by Western blot, both with an antibody against SCL and with Streptavidin to detect the biotinylated protein. Three clones were expanded and tested for SCL function. The percentage of Flk1-expressing cells increased, while that of PDGFRα expressing cells significantly decreased in response to 48 hours of SCL expression on days 3 and 4 of EB differentiation (Fig 4.7B). Therefore, I concluded that the biotinylated protein functioned as wild-type SCL in early mesoderm.

Both iSCL and iAvi-SCL cells were induced for 2 days starting on day 2 and protein was isolated on day 4. In silver stained gels the SCL band was clearly visible. 6 additional bands specific to iAvi-SCL were cut and sent for sequencing by mass spectrometry. Only one of those bands was identified. This turned out to be LMO2. No specific peptides were identified in the remaining bands. (Fig 4.8) Work is ongoing to identify these proteins.

d. Transcriptional targets of SCL in the mesoderm patterning stage EBs

The EB system is well suited for high throughput analysis, because it can provide large amount of starting material. Once I had established the role of SCL in the model of early development, I performed a microarray analysis to find new transcriptional targets of the protein during early development.

For microarray analysis, d2.5 iSCL EBs were induced for 6 hours before RNA collection. The 6 hour time point was chosen to limit the findings to direct targets of SCL. qRT-PCR (Fig 4.9A) and western blotting (Fig 4.9B) showed that SCL is already expressed after a short induction.

Figure 4.7

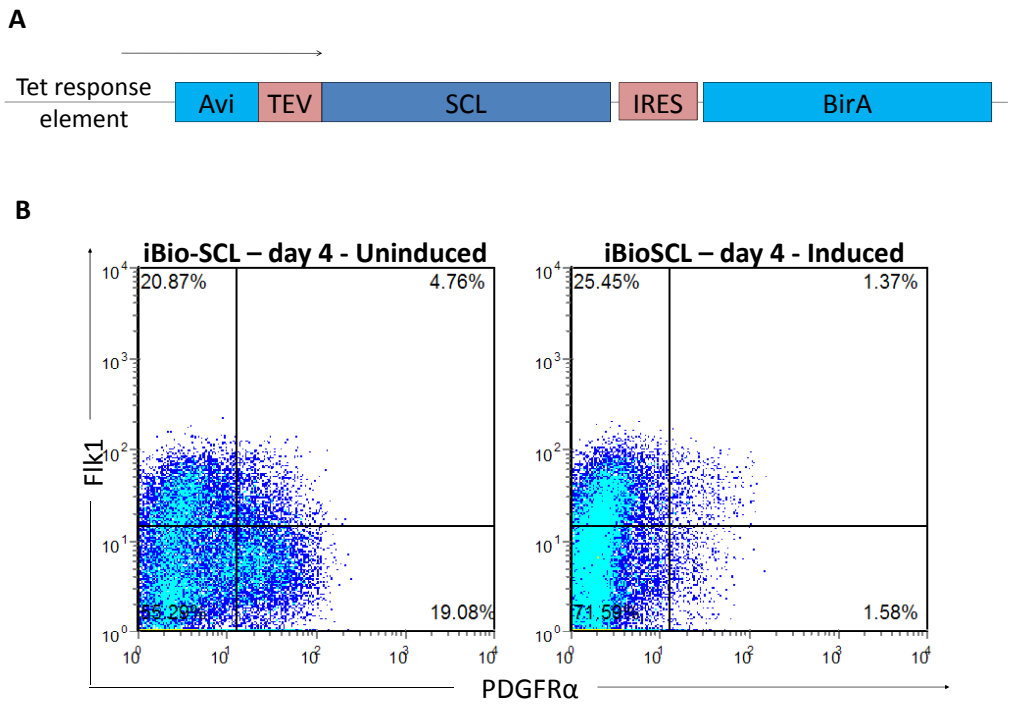


Figure 4.7: **A)** Schematic representation of the iBio-SCL locus. Avi: Biotinylation sequence. TEV: TEV protease cleavage sequence. IRES: Internal Ribosome Entry Site. BirA: Biotin ligase. **B)** Mesoderm patterning activity of Bio-SCL assessed based on the expression of Flk1 and PDGFRα in the uninduced and induced cells.

Figure 4.8

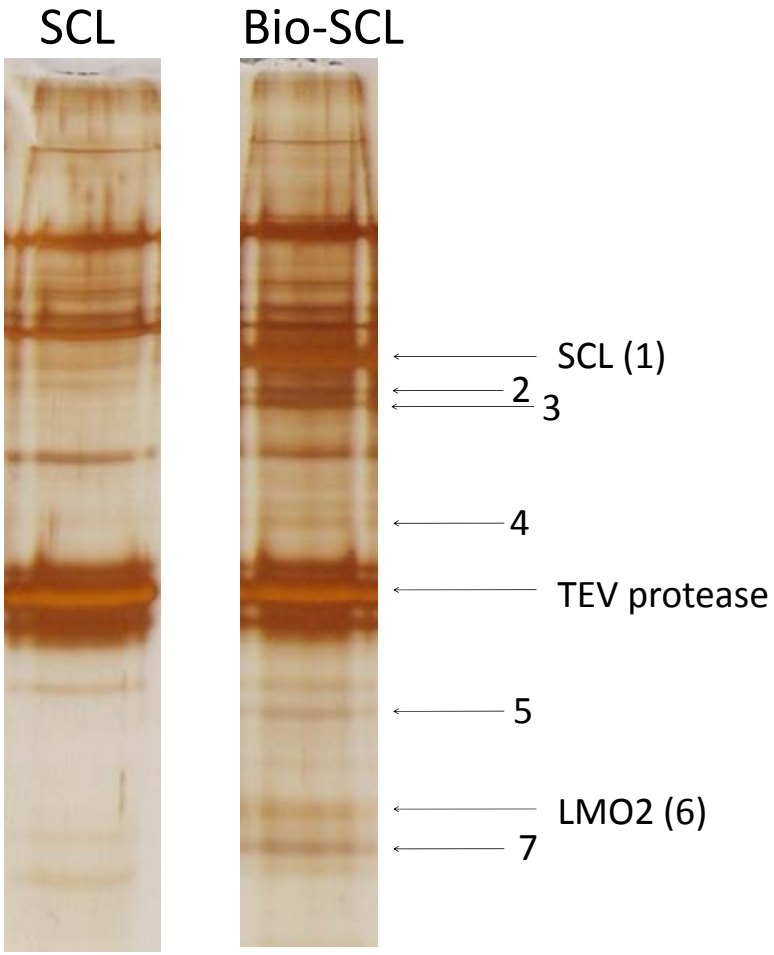


Figure 4.8: Silver staining after IP with Streptavidin beads and elution by TEV cleavage. Bands specific to iBioSCL are numbered. All bands except for SCL and LMO2 remain unidentified so far.

A small number of genes were regulated in response to short term SCL induction. Of these, five biologically relevant targets, Chordin, FoxA2, Id1, Id2 and Id3 were selected and the change in the expression levels was confirmed using qRT-PCR on three independent biological samples (Fig 4.9C).

I compared the repression of the newly identified SCL target genes in response to SCLF238G and SCLRERAAA expression in order to determine LMO2 and DNA binding dependent targets (Fig 4.10). The change in the level of gene expression of all targets was lower in response to SCLF238G overexpression, suggesting a requirement for LMO2 binding.

e. SCL induces proximal mesoderm in the absence of serum factors

In vitro differentiation towards mesoderm requires serum factors. However, composition of the serum is not constant among different lots. In order to be able to control the experimental conditions more precisely, I decided to replace serum with KOSR produced by Invitrogen (Cat #10828-018). I hypothesized that SCL expression by itself would not be able to induce lateral plate mesoderm in these cultures, since there will not be any mesoderm to pattern.

Surprisingly, the expression of SCL during days 3 and 4 in EBs, grown in serum free EB-diff, led to formation of whole mesoderm judged by FACS analysis on day 4 (Fig 4.11A). The presence of the hematopoietic cells in day 6 EBs was confirmed by CFC assay and RT-PCR (Fig 4.11B,C). In addition to the hematopoietic differentiation, 3 out of 12 EBs plated for cardiac differentiation initiated autonomous beating in culture, proving formation of a non-hematopoietic lineage, cardiac, in response to SCL

Figure 4.9

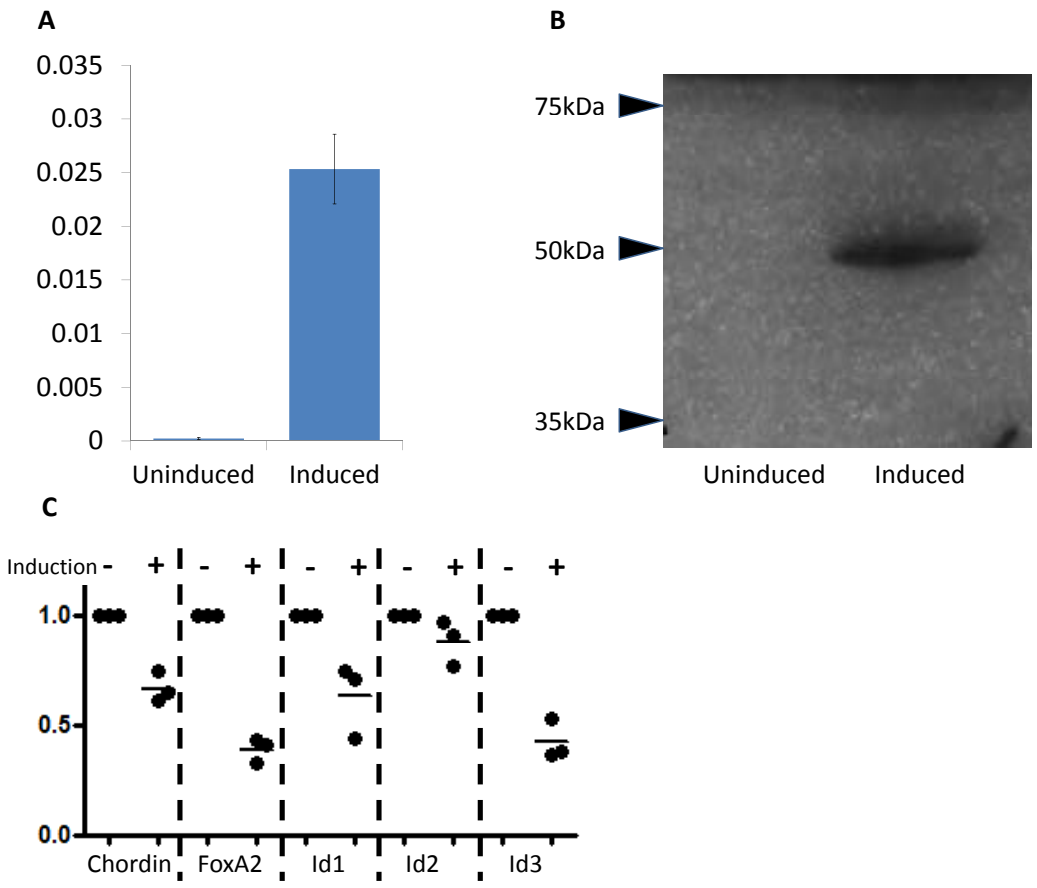


Figure 4.9: Analysis of SCL expression 4 hours after induction by **A)** qRT-PCR and **B)** Western blot with α-SCL antibody (BTL73). Expression of candidate target genes in response to 4 hours of SCL induction. **C)** Confirmation of the downregulation of target genes. Level of expression of five SCL target candidates were analyzed in three independent sets of uninduced and 6-hour induced day 2.5 EBs. Levels of expression are shown as the fold of uninduced control.

Figure 4.10

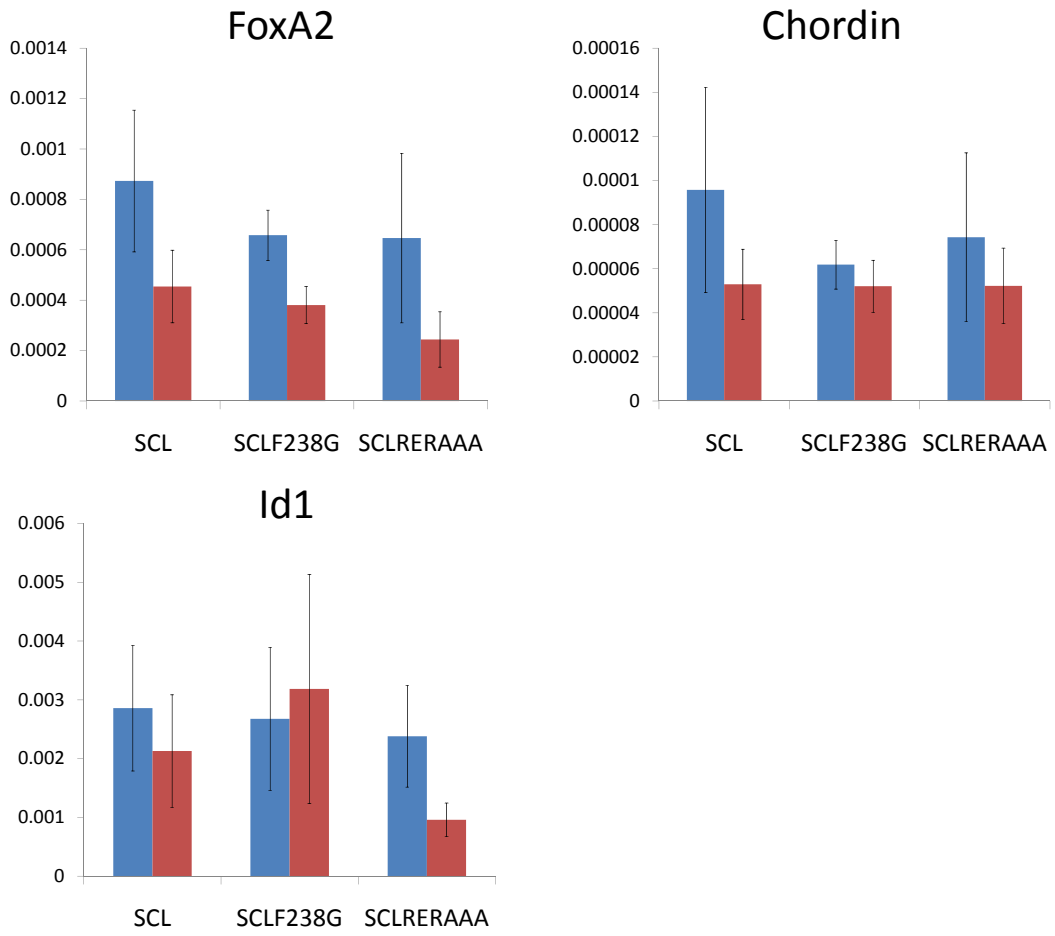


Figure 4.10: Change in the level of SCL target gene expression in response to 6 hours of SCL, SCLF238G or SCLRERAAA induction. Levels shown are fold of GAPDH. Blue bars represent uninduced, red bars represent induced samples. Error bars show the standart error from three biological replicates.

Figure 4.11

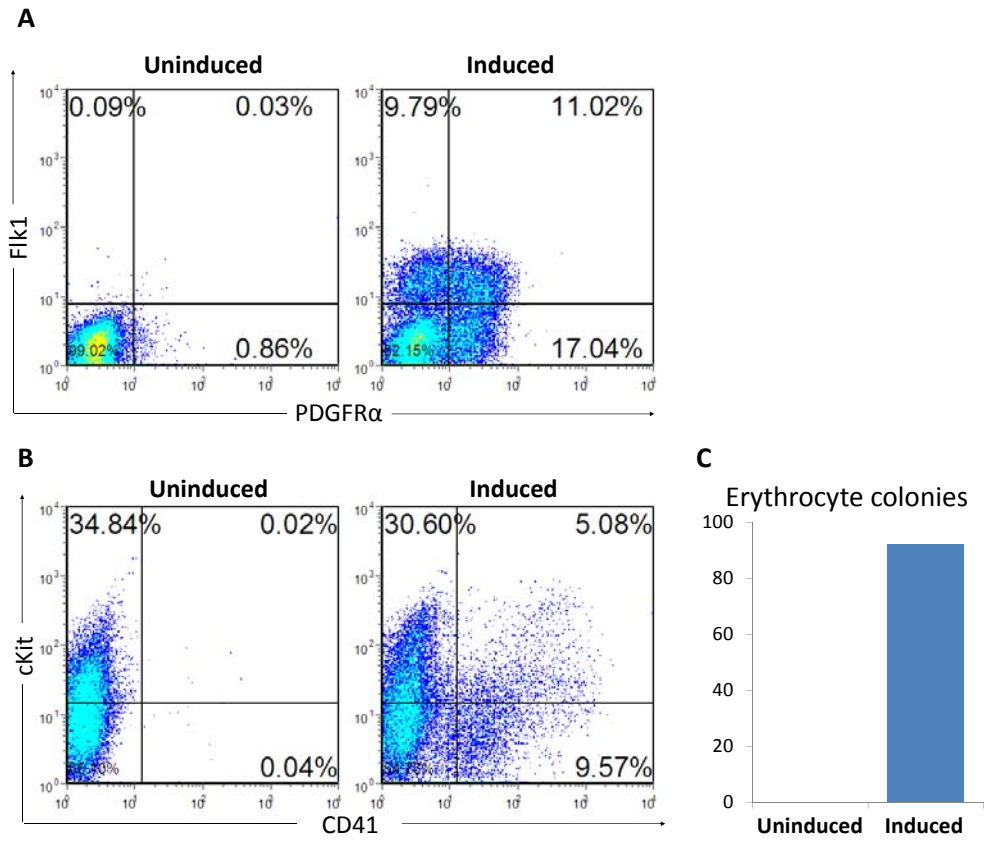


Figure 4.11: Induction of proximal mesoderm by SCL under serum-free conditions. **A)** Surface phenotype of day 4 EB cells. **B)** Surface phenotype of day 6 EB cells. **C)** Erythrocyte colonies obtained from 50000 day 6 EB cells.

expression under serum-free conditions. Markers of the skeletal muscle lineage were not expressed at significant levels.

Discussions and Conclusions

I have obtained three other lines of additional evidence supporting the model of SCL-LMO2 interaction in early hematopoiesis: The lack of a mesoderm patterning effect of the SCLF238G mutant, co-immunoprecipitation of LMO2 with a tagged SCL protein and decreased repression of SCL target genes after the expression of SCLF238G.

The absence of a mesoderm patterning effect in response to ectopic LMO2 expression seems to be against prior evidence suggesting an early mesoderm stage interaction with SCL. Most of the LMO2 protein in normal cells is bound to SCL and the half life of the unbound LMO2 is significantly shorter than molecules bound to SCL (Lecuyer et al., 2007). Therefore, the overexpressed LMO2 might be degraded quickly.

The data I obtained in GATA2 ectopic expression experiments contradicts the paper by Lugus and colleagues (Lugus et al., 2007). They found GATA2 to be a hemangioblast enriched gene in a microarray analysis, expressed it ectopically in differentiating EBs under serum free conditions and showed that it can produce $Flk1^+$ and SCL^+ mesoderm. The main difference between the two techniques is in the preparation of the EBs. We used the hanging drops method, which starts with 100 cells per EB, while they employ the methylcellulose method where each EB comes from a single cell. A change in timing of differentiation could mean that we are observing the effect of GATA2 expression at different stages.

The change in the levels of expression of the target genes was limited in the microarray experiment. This might be due to the short window of expression I have opted to use. My reason for using a 6 hour window was to detect direct targets of SCL rather than proteins regulated due to secondary effects like the decrease in paraxial mesoderm or the increase in lateral plate mesoderm progenitors. Dox dependent gene expression reaches near maximal levels as early as two hours after the induction in our system. However, most of the targets identified were downregulated genes and therefore the half life of the RNA that is already in the cell before the induction affects the level of downregulation.

An incomplete repression of some or all target genes by SCL is theoretically possible. SCL might be competing out the transcription factors responsible for the expression of these genes. Also, expression in cells that do not express LMO2 might escape SCL mediated repression. Finally, complete repression might require chromatin remodeling, which may not be finished in 6 hours.

FoxA2 (Kinder et al., 2001a) and Chordin are both expressed in the distal primitive streak. SCL, by downregulating this transcription factor might be suppressing distal fates (node, somites) in favor of the proximal hematopoietic mesoderm. The transcriptional repression of these genes by SCL is supported by their increased expression in the SCL-/- EBs.

Id1 and Id3, members of the Id protein family, can bind to HLH transcription factors, but cannot bind to DNA. Therefore they are known as inhibitors of the HLH family. Id proteins have been shown to interfere with the differentiation of the muscle

when overexpressed (Jen et al., 1992). Their downregulation by SCL thus suggests a positive feedback mechanism that reinforces the effect of SCL expression.

A mesoderm inducing role for SCL is unexpected based on the normal gastrulation phenotype of knockout mouse embryos (Elefanty et al., 1999; Robb et al., 1995; Shivdasani et al., 1995) and contribution of the knockout cells to all tissues in chimeric embryos (Porcher et al., 1996; Robb et al., 1996). Also, my qRT-PCR analysis showed no effect of SCL expression on Brachyury expression in serum containing medium (Fig 3.5B). These data suggest that the mesoderm inducing effect of SCL is a secondary phenotype, which is unmasked by the absence of the regular mesoderm-inducing factors.

To explain these observations I propose a model, where SCL lowers the threshold of cells to BMP4 signal either by cell-autonomous mechanism or by repressing the competing distal primitive streak fate, which decreases the effect of BMP4 signal through secretion of inhibitors, like Chordin (Fig 4.12). Mesoderm induction is not affected in the SCL-/- embryos, because of high amount of BMP4 secretion. In other words, suppression of the distal primitive streak by SCL is only a secondary mechanism in development. Still, the increased expression of FoxA2 and Chordin in SCL-/- EBs points to the possibility of an undiscovered perturbation in the primitive streak of these embryos. More careful analysis of E6.0-E7.5 embryos is required for a definitive answer to this question.

Figure 4.12

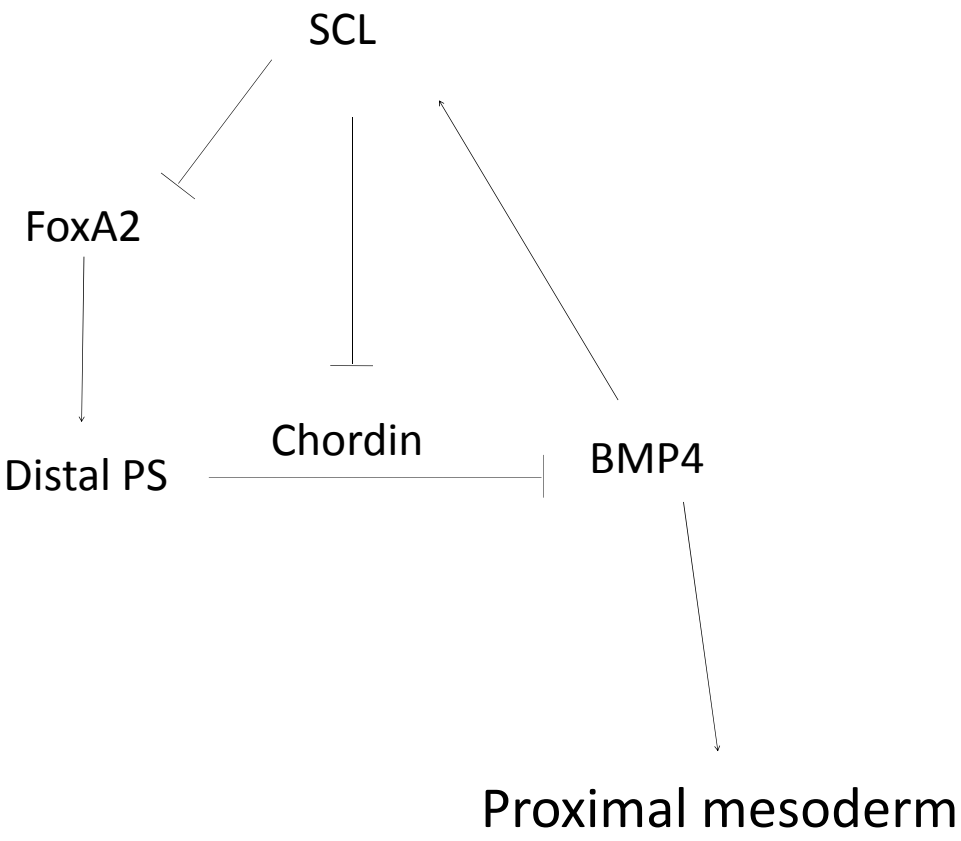


Figure 4.12: Proposed model of mesoderm patterning by SCL.

CHAPTER 5: CO-EXPRESSION OF SCL COMPLEX MEMBERS SCL, LMO2 AND GATA2 INDUCE EFFICIENT HEMATOPOIESIS IN MONOLAYER CULTURE IN THE ABSENCE OF SERUM

Introduction

The formation of the primitive streak in the posterior epiblast and its patterning to blood requires induction by the visceral endoderm (Belaoussoff et al., 1998). The most common method for hematopoietic differentiation *in vitro* is EB formation. EBs contain both epiblast-like and visceral endoderm-like populations that can interact and mimic the mesoderm induction in the embryo (Doetschman et al., 1985). Therefore, ES cells differentiate to blood readily when cultured in EBs (Keller et al., 1993). Another requirement for blood formation is the mesoderm-inducing activity of FBS. EBs differentiated in serum-free media without added growth factors will form a small amount of mesoderm, probably due to paracrine signaling, but no blood (Johansson and Wiles, 1995).

The signaling factors present in each lot of FBS may vary significantly and affect the results obtained in different labs. Furthermore, variation in intracellular interactions in EBs may even affect different experiments in the same lab. The monolayer culture system is better suited to isolate and investigate the effect of one factor, because the control of the experiment is more precise. Production of blood in a serum-free monolayer culture system would also be preferred in a clinical setting, since it avoids the use of an animal product, FBS and is easy to scale up for large quantities.

Emrbyonic development drives cells to a state where specific gene sets that are necessary for the function of a specific cell type are expressed, while the expression of most other genes is silenced. As cell fates become more specialized, more permanent epigenetic marks of chromatin, e.g. histone deacetylation, phosphorylation and methylation, are used to silence the unused portion of the genome.

An attractive approach to bypass the complexity of developmental pathways is to modulate the transcriptome directly with transcriptional regulators that govern the lineage of interest (Darabi et al., 2008). Candidates that might fit this description and drive cells to a hematopoietic fate have been identified through the study of hematopoietic development in embryos.

Ihh, a signal molecule secreted by the visceral endoderm, can induce hematopoiesis (Dyer et al., 2001). Ihh upregulates BMP4 expression and BMP4 has been shown to induce hematopoietic factors SCL (Mead et al., 1998), LMO2 (Mead et al., 2001) and GATA2 (Maeno et al., 1996) in *Xenopus* embryos. Expression of SCL or GATA2 is sufficient for blood formation in animal explants (Maeno et al., 1996; Mead et al., 1998).

In this part of the study, I used ectopic SCL expression to induce blood formation in a serum free monolayer system. I observed that while expression of SCL alone is capable of driving a small number of cells to the hematopoietic lineage, co-expression of SCL, LMO2, and GATA2 induces this lineage rapidly and in the majority of the cells. When the three factors are co-expressed, induction of blood does not proceed through the regular mesodermal route, requires only 24 hours of ectopic expression and is complete in 48 hours. The results suggest, progenitors fated for the ectodermal lineage are stably converted to blood cells in response to SCL, LMO2, GATA2 cocktail expression.

Results

a. SCL can induce hematopoiesis in monolayer culture

First, I followed the expression of the key differentiation markers in the monolayer differentiation of the E14 cells. Pluripotency markers Oct4, Nanog and Rex1 were quickly downregulated. Fgf5, a marker of epiblast, was initially upregulated and reached its peak on day 2 (Fig 5.1A). Expression of mesendodermal markers was low compared to the EBs grown in serum. This was also apparent from the lack of PDGFRα/Flk1 expression in the uninduced cultures (Fig 5.2A). All mesodermal markers analyzed peaked on day 4 of culture (Fig 5.1B). Ectodermal markers were expressed most robustly in this culture system. Their expression continued to increase until day 6, the last day of analysis in this experiment (Fig 5.1D).

I grew the iSCL cells in monolayer cultures and ectopically induced the expression of SCL. $Flk1^{+}$ cells emerged in the induced wells on day 4 of culture (Fig 5.2B). On day 6, the analysis of hematopoietic markers by FACS and colony assays showed the production of the hematopoietic cells only in the induced wells (Fig 5.3A-B).

b. Co-expression of SCL, LMO2 and GATA2, allows production of the blood cells from the pluripotent cells with high efficiency

SCL was shown to form a protein complex in hematopoietic cell lines. The interaction with proteins LMO2 and GATA1 or GATA2 maximizes the transcription from a reporter plasmid, which contains E boxes and GATA binding sites (Wadman et al., 1997). In zebrafish, it was shown that SCL's ability to promote hematopoiesis was limited to the tissues where it can induce LMO2 expression and co-expression of the two

Figure 5.1

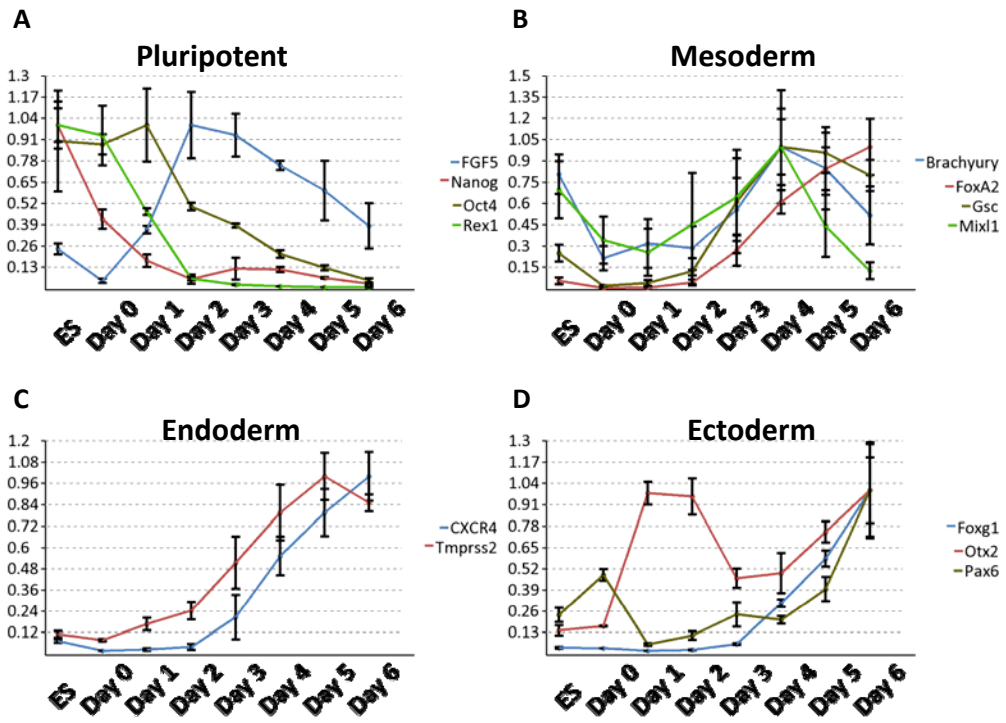


Figure 5.1: Analysis of differentiation in monolayer cultures. Genes specific for four embryonic lineages were analyzed by qRT-PCR. Levels were normalized to the maximum in the set for each marker to show trends. The error bars show the standard error from three biological replicates.

Figure 5.2

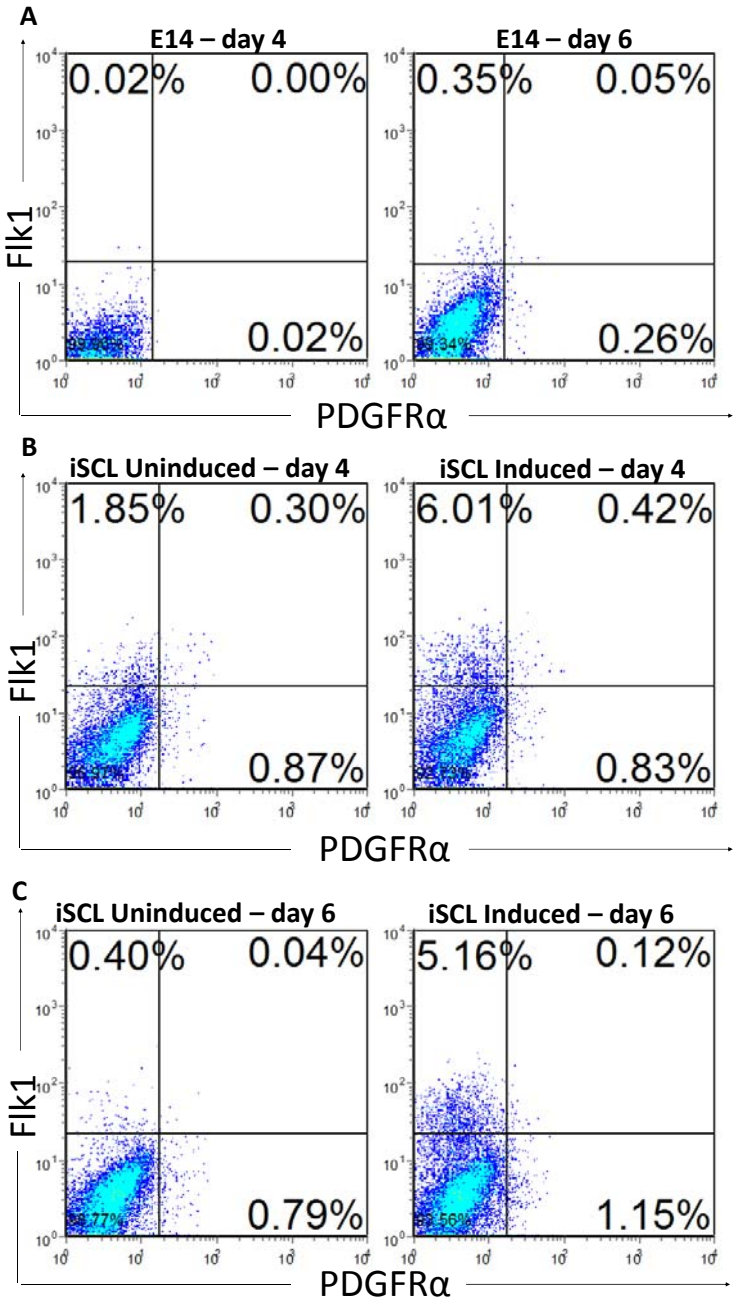


Figure 5.2: Mesoderm differentiation in monolayer cultures. Analysis of mesoderm differentiation from **A)** E14 and **B-C)** iSCL cells in monolayer culture by FACS.

Figure 5.3

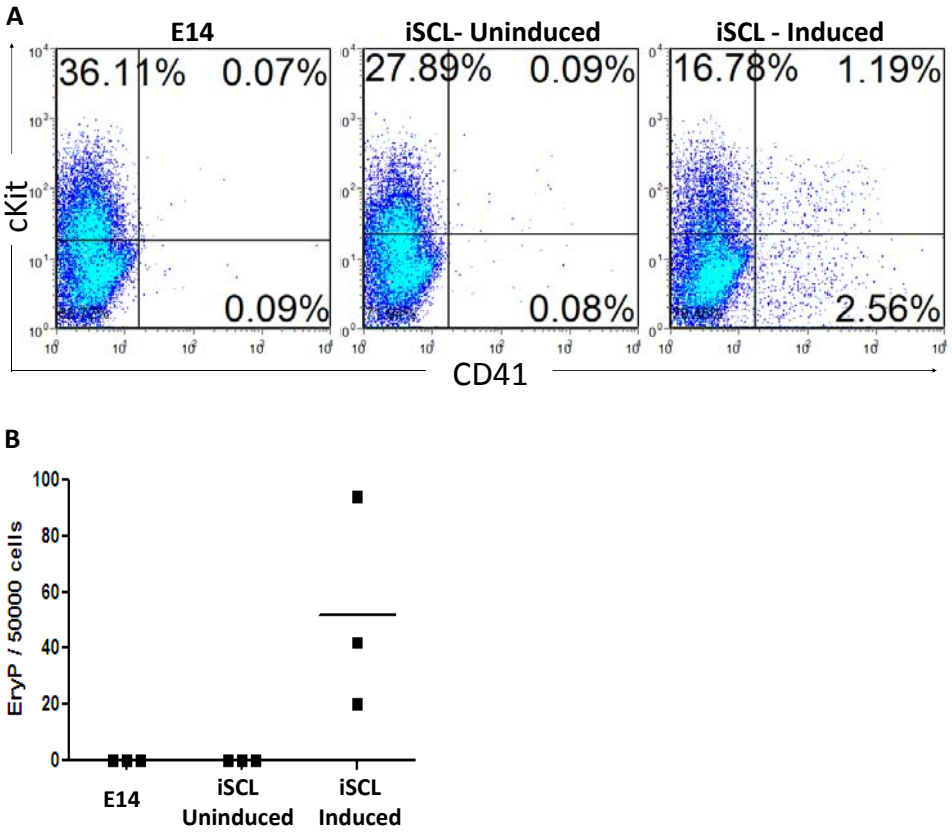


Figure 5.3: Induction of hematopoiesis by SCL in monolayer culture. **A)** FACS analysis of day 6 E14 and iSCL cells. **B)** EryP colonies obtained from 50000 differentiated cells.

proteins was more effective in inducing blood from non-hematopoietic tissues like the cardiac or the somitic mesoderm (Gering et al., 2003). Also, the expression of SCL and LMO2, with the addition of GATA1 has been shown to induce ectopic blood formation in the zebrafish (Gering et al., 2003) and the *Xenopus* (Mead et al., 2001) embryos.

Increase in hematopoietic differentiation efficiency in response to co-expression of SCL and LMO2 was only marginal (Fig 5.4). I therefore tested the effect of co-expressing SCL, LMO2 and GATA2.

I noted a striking morphological change in response to the transcription factor cocktail expression (Fig 5.5A). Many cells in the induced cultures adopted a round morphology and then detached from the plate, suggesting a hematopoietic fate. The suspension cells were almost homogenously $CD41^+$ (Fig 5.5B), but they had very low hematopoietic colony forming potential.

Efficient induction of hematopoiesis by day 6 of differentiation was confirmed by the expression of the hematopoietic marker CD41 (Fig 5.6A). I evaluated Flk1 and PDGFRα expression on day 4 to determine whether *in vitro* hematopoiesis was preceded by a wave of mesoderm. Although the number of the Flk1 expressing cells was slightly higher in the induced set, the increase was miniscule compared to the levels of CD41 expression two days later (Fig 5.6B). Unexpectedly, the increase in the number of CD41-expressing cells did not translate to an increase in colony forming ability relative to that seen with SCL alone under these conditions of continual induction (Fig 5.6C).

c. 24 hour expression of the transcription factor cocktail is sufficient for programming

The SCL complex is involved in the differentiation and maturation of erythrocytes and megakaryocytes and thus I reasoned that maintained ectopic expression

Figure 5.4

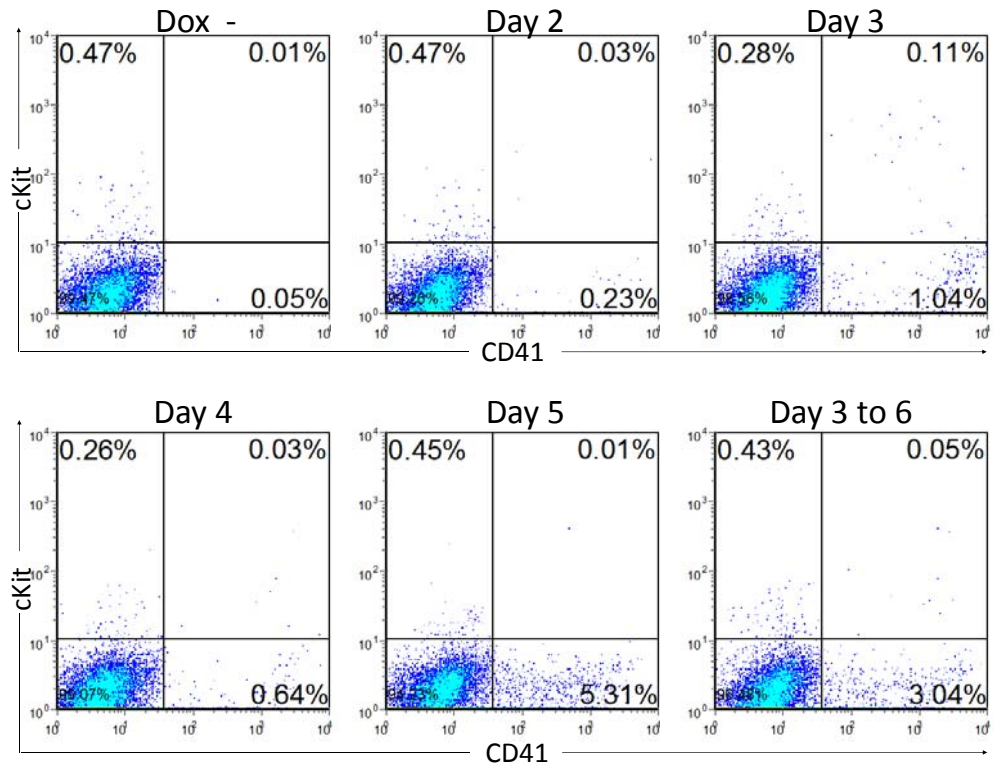


Figure 5.4: Hematopoietic differentiation in response to SCL-LMO2 expression. The cells were induced on the noted day for 24 hours, except for the last sample, which was induced for 4 days. Surface phenotype was analyzed on day 6.

Figure 5.5

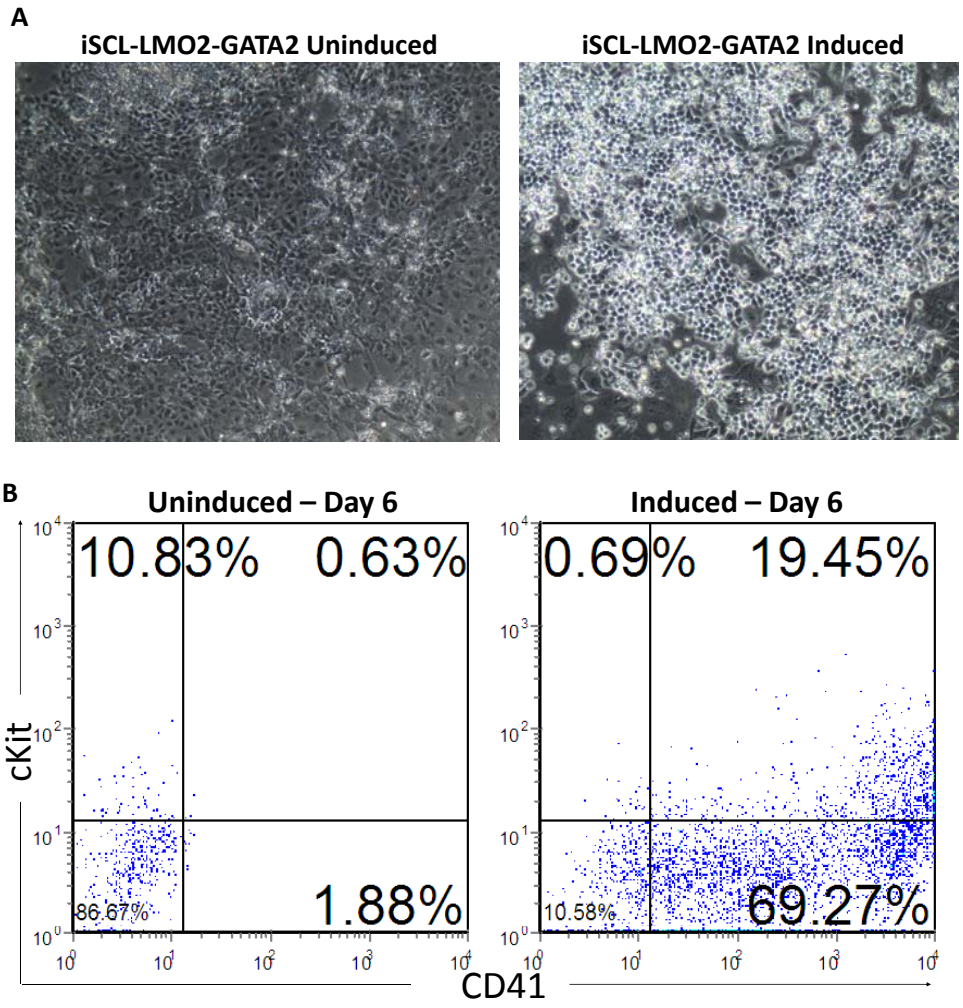


Figure 5.5: Induction of hematopoiesis by SCL, LMO2 and GATA2 coexpression. **A)** Morphological change observed in monolayer cultures in response to SCL-LMO2-GATA2 expression. **B)** Surface phenotype of the cells that detach from the plate after induction.

Figure 5.6

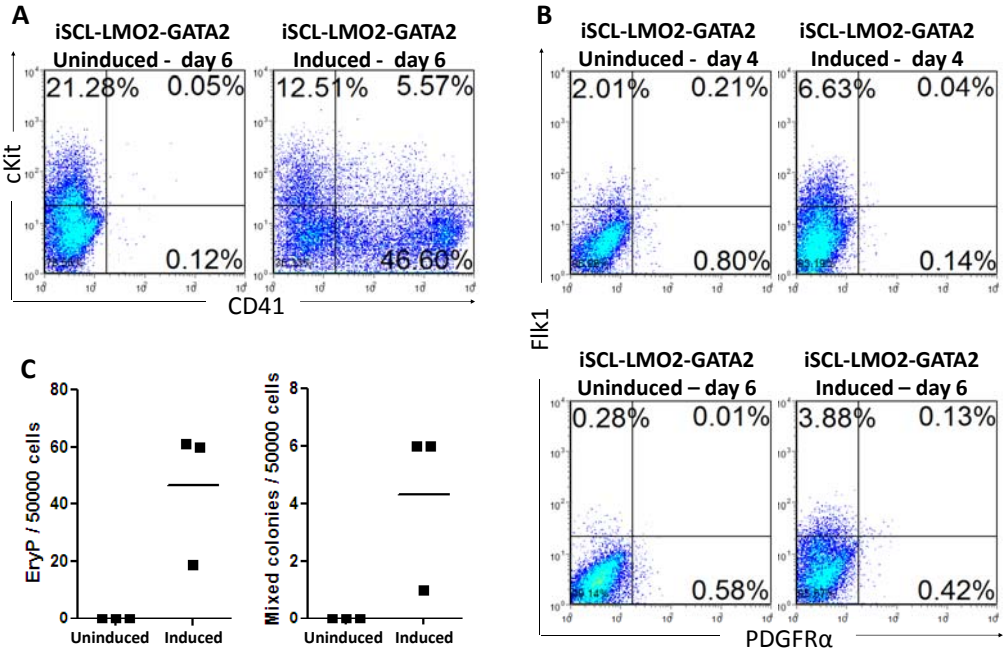


Figure 5.6: Induction of hematopoiesis by SCL, LMO2 and GATA2 coexpression. **A-B)** Surface phenotype of cells differentiating as monolayer. **C)** Hematopoietic colonies obtained from 50000 iSCL-LMO2-GATA2 cells.

of these factors might be driving the cells towards terminal differentiation once they are committed to hematopoietic fate. Also, long term culture of hematopoietic progenitors requires supportive cytokines or feeder cell lines, which are absent from this culture system. As a result, hematopoietic progenitors might be differentiating rapidly after being generated. I therefore tested the effect of inducing the factors in 24 hour pulses. SCL, LMO2 and GATA2 were induced on different days of differentiation and all samples were analyzed on day 6 for the expression of the CD41 marker and for colony forming potential. Induction on day 5 was the most potent by both indicators (Fig 5.7 A-B). Consistent with the idea that maintained over-expression of SCL, LMO2 and GATA2 might have been forcing the subsequent differentiation of the hematopoietic progenitor cells produced in monolayer; I observed significant colony forming activity under these conditions. When the cells induced during day 5 were plated in semisolid medium on day 6, about 300 erythroid colonies formed from 50000 cells (Fig 5.7B). More significantly, whereas previously with longer inductions, we observed only a few multi-lineage colonies, the short induction generated about 100 mixed-lineage colonies per 50000 cells, indicating the presence of progenitors higher in the hematopoietic hierarchy (Fig 5.7B).

Hematopoietic cells are normally cultured in the presence of supporting cytokines or feeder cell lines neither of which is present in our culture system. Therefore, a loss of potential might be expected when the cells spend time in culture after being programmed to hematopoietic lineage. To test this, the 24 hour induction time course was repeated with the analysis of CD41 expression quickly afterwards. Since the change in morphology was first observed 48 hours after the beginning of the Doxycycline administration, I concentrated my analysis on this time point.

Figure 5.7

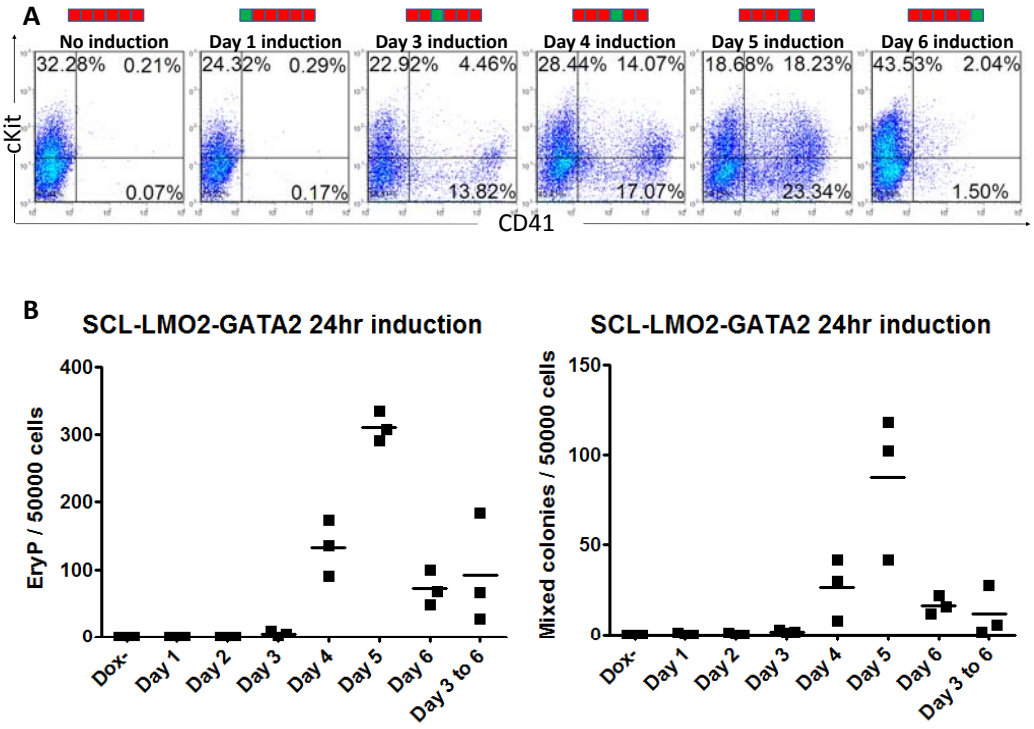


Figure 5.7: 24 hours of factor expression is sufficient for hematopoietic induction. **A)** FACS profile of iSCL-LMO2-GATA2 cells induced for 24 hours at different time points during culture and analyzed on day 6. Each box represents a day of culture, green boxes represent the day of induction. **B)** EryP and mixed lineage colony counts from day 6 iSCL-LMO2-GATA2 cultures.

SCL, LMO2 and GATA2 were expressed for 24 hours during different days of monolayer culture and cells were analyzed 24 hours after the removal of Doxycycline (Fig 5.8A). Only about 2% of the cells were $CD41^+$ when cultures were induced on the first day of differentiation and analyzed at the end of the second day. Induction during the second day yielded higher percentage of $CD41^+$ cells. After this initial period of weak response, induction resulted in about 40%-50% conversion to the hematopoietic lineage. The efficiency of the hematopoietic programming decreased again when cells were induced after 9 days of culture. From these data, we concluded that the cells are most responsive to the hematopoietic programming factors between days 3 and 9 of the culture.

I further characterized hematopoietic programming using day 5 induction. 24 hours after the beginning of the induction, only limited CD41 expression was seen, indicating low level of hematopoiesis (Fig 5.8B). The percentage of the $CD41^+$ cells reached maximum levels (~40-50%) at 48 hours (Fig 5.8C). At this time point, expression of globin genes was also much higher than at 24 hours (~80 fold increase for embryonic globin and ~400 fold increase for β-major globin) (Fig 5.8D).

d. Programming does not proceed through a mesodermal intermediate

To see if the cultures were programmed to the hematopoietic fate through a mesodermal precursor, I compared the expression of mesodermal marker Brachyury in the uninduced and induced cultures 24 and 48 hours after the beginning of the induction. The level of Brachyury expression was significantly lower in the induced cultures at both time points (Fig 5.9A). This finding rules out a mesodermal intermediate in the induced hematopoiesis of these cells. The decrease in the level of Brachyury suggests that

Figure 5.8

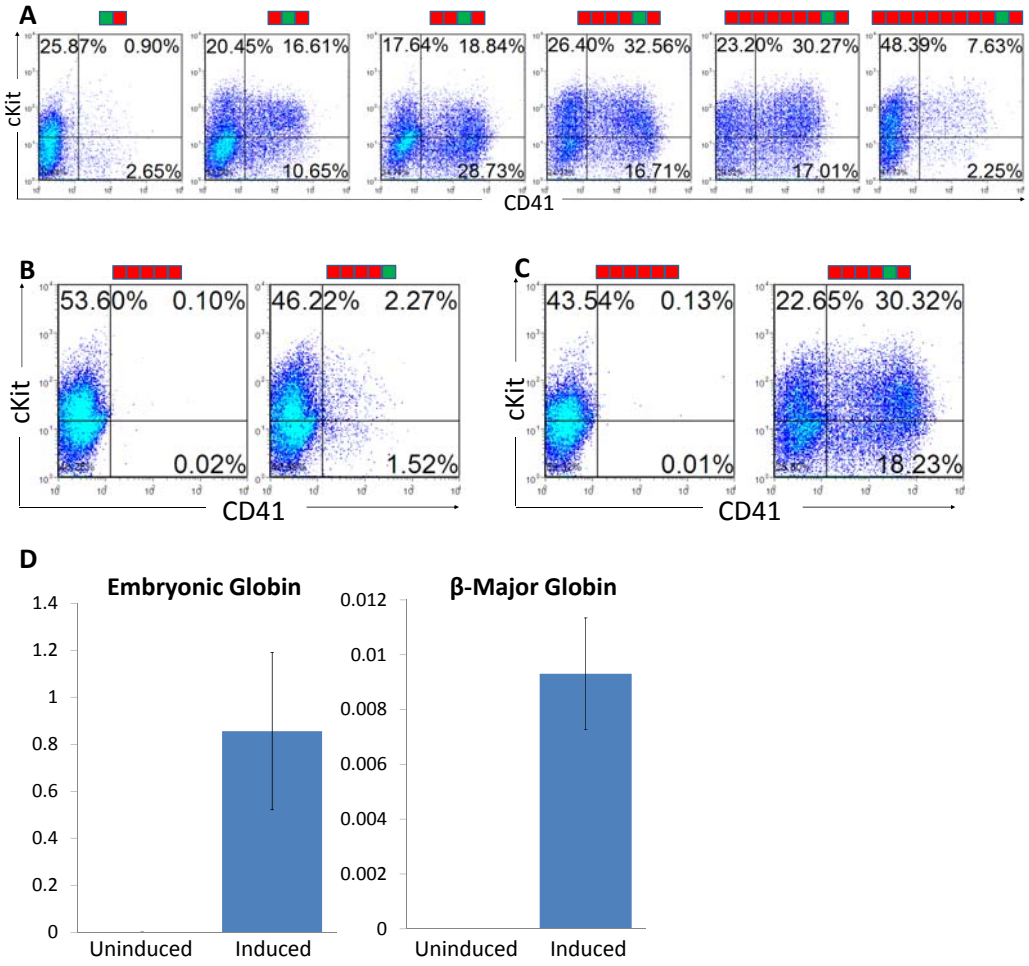


Figure 5.8: Hematopoietic programming is completed in 48 hours. **A)** iSCL-LMO2-GATA2 cells were induced for 24 hours at different days during culture and surface profile analyzed 48 hours later. Emergence of hematopoietic cells after 48 hours shown by **B-C)** FACS analysis and **D)** qRT-PCR analysis of globin expression. The levels shown are fold of GAPDH. The error bars represent standard error from three independent biological replicates.

mesodermal precursors differentiated quickly in response to SCL, LMO2 and GATA2 expression. However, considering low levels of Brachyury (Fig 5.1B) and Flk1 (Fig 5.5B), a model where transcription factors only act on mesoderm cells cannot explain the conversion of half of the cells in the culture to hematopoietic fate in only 48 hours.

e. Programming to the hematopoietic fate is irreversible in ectoderm

To obtain insight into the nature of the cells that were responding to the transcription factor cocktail, I measured the expression of two ectodermal transcription factors Pax6 and Sox1, and mesendodermal marker FoxA2 in the SCL-LMO2-GATA2 uninduced and induced cultures. In only 24 hours, expression of these markers was decreased 5 to 10 fold (Fig 5.9). To determine whether this loss of marker expression was not due to transient repression but indicated a stable change of fate, I analyzed the cultures after the removal of induction. SCL-LMO2-GATA2 RNA was expressed for 24 hour periods during differentiation and all samples were collected and analyzed on day 6.

Expression of Fgf, Brachyury and Goosecoid recovered to near basal levels after the removal of Dox from the cultures, suggesting many progenitors that will give rise to Brachyury-expressing cells are not affected by the inducing cocktail (Fig 5.10). On the other hand, the expression of the ectodermal markers Pax6, Foxg1, Ngfr and Sox1 were still low even 4 days after the removal of the induction (Fig 5.10). These data show that ectopic expression of SCL complex proteins SCL, LMO2 and GATA2 for 24 hours reprogrammed ectodermal progenitors to the hematopoietic lineage irreversibly.

Figure 5.9

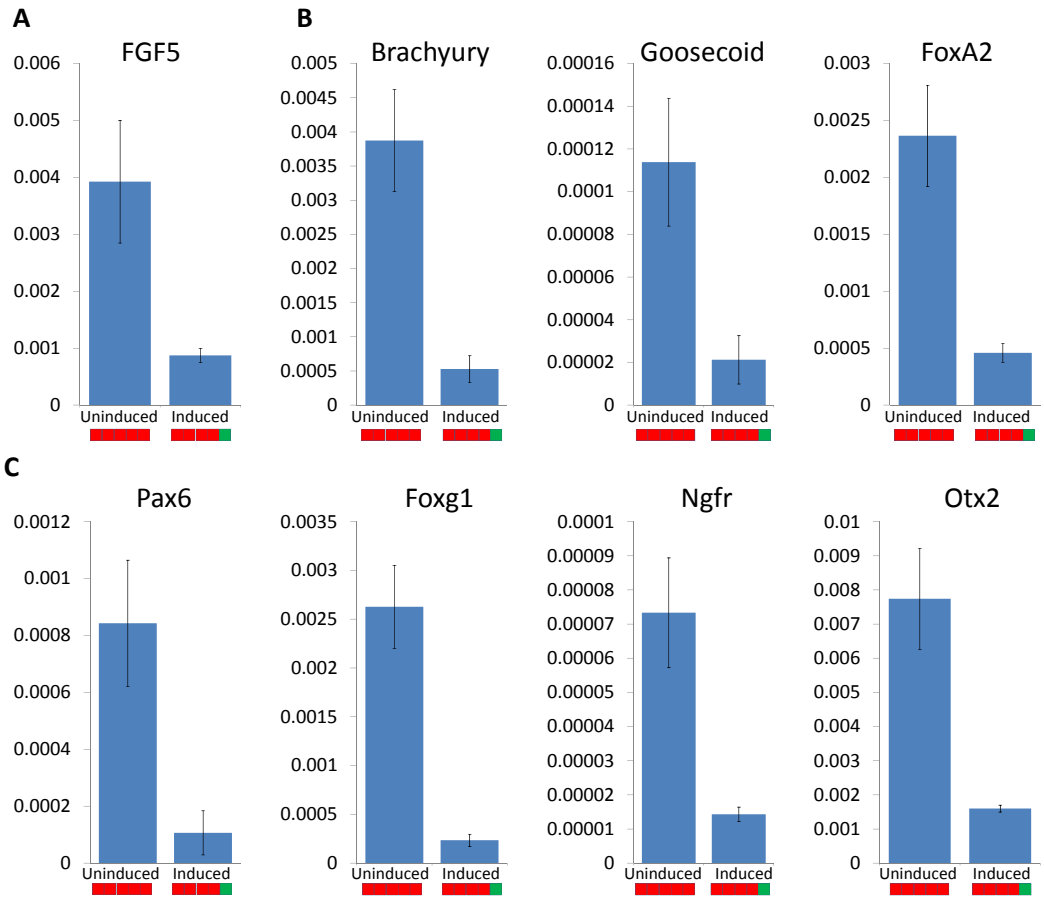


Figure 5.9: qRT-PCR analysis of **A)** epiblast, **B)** mesendodermal and **C)** ectodermal marker genes in day5 iSCL-LMO2-GATA2 cells. The induced cells received Dox for 24 hours right before the collection of the RNA. The levels shown are fold of GAPDH. The error bars represent standard error from three independent biological replicates.

Figure 5.10

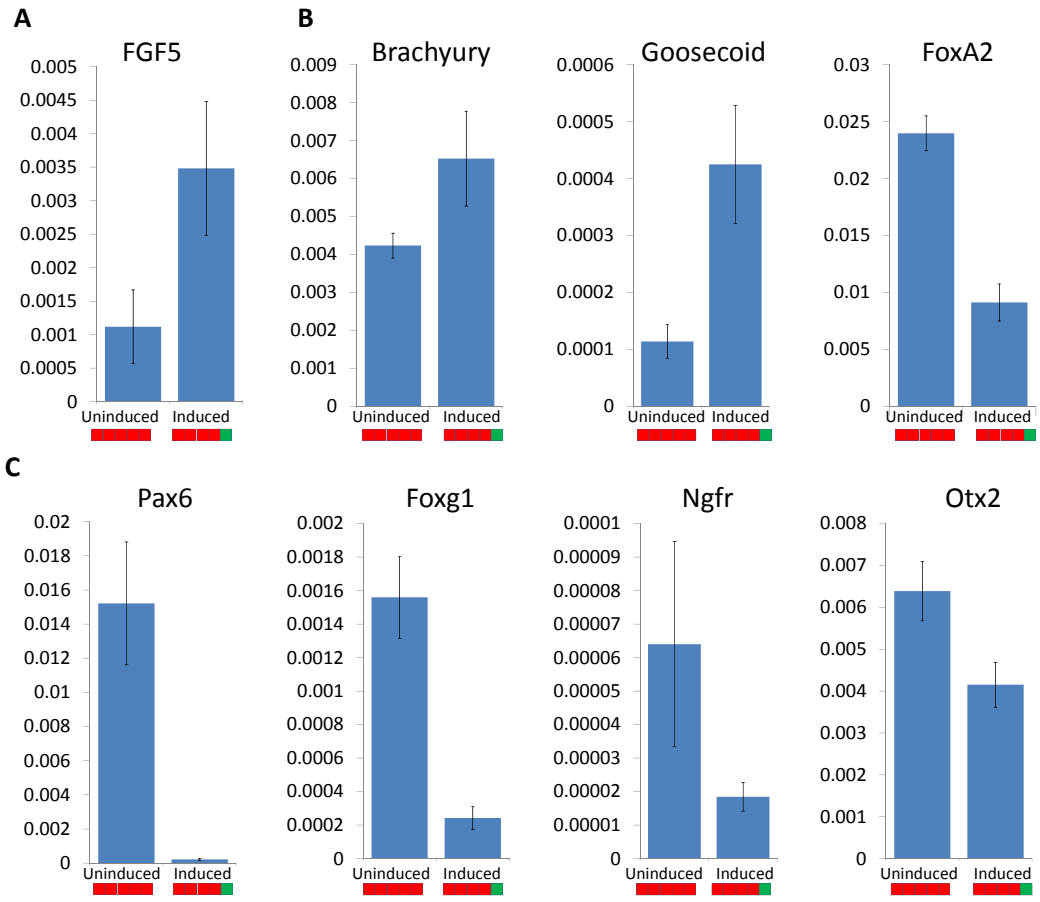


Figure 5.10: qRT-PCR analysis of **A)** epiblast, **B)** mesendodermal and **C)** ectodermal marker genes in day6 iSCL-LMO2-GATA2 cells. The induced cells received Dox for 24 hours on day 3 of culture. The levels shown are fold of GAPDH. The error bars represent standard error from three independent biological replicates.

Discussion and Conclusions

In this part of my study, I have shown the ability of a three member transcription factor cocktail to directly induce hematopoiesis from non-mesodermal cells in a monolayer, defined medium culture system in the absence of signaling factors that are normally required for blood development.

I have previously shown that expression of SCL in EBs can skew differentiation towards hematopoiesis. In this serum free monolayer system, SCL alone was able to induce some Flk1 expression and hematopoiesis. However, the levels were much lower compared to the EBs cultured in serum containing medium. From this I concluded that the effect of SCL was limited to the low number of mesoderm cells created in this system. SCL has been shown to be a part of a self regulating hematopoietic transcriptional network with GATA2 and Fli1 (Pimanda et al., 2007), but these data show that SCL expression by itself is not sufficient to start the cascade.

Studies in model organisms showed co-expression of LMO2 extended the area programmed to blood in response to SCL expression (Gering et al., 2003; Mead et al., 2001). In our experiments, expression of two factors together did not improve the output significantly. The dramatic improvement in hematopoietic programming was only observed after the addition of GATA2 into the cocktail.

Previous studies in zebrafish (Gering et al., 2003) and *Xenopus* (Mead et al., 2001) models used GATA1 instead of GATA2 and observed ectopic erythropoiesis rather than multilineage hematopoiesis. GATA1 is normally limited to the erythropoietic lineage and thus may not support other progenitors (Ohneda and Yamamoto, 2002). Also, in the experiments cited, the ectopic expression of the factors was not turned off. As we

have observed, continuous expression of these factors leads to rapid differentiation instead of progenitor expansion. Interestingly, Gering and colleagues co-expressed GATA1 instead of GATA2 with SCL and LMO2 in their *Xenopus* experiments, but this resulted in the loss of erythropoiesis induction phenotype (Gering et al., 2003). There are hematopoiesis-blocking signals in an embryo, as well as inducing ones. The programming potential of the SCL complex might have been masked in the complex environment of an embryo.

GATA2 is known to be necessary for the proliferation of early hematopoietic progenitors (Tsai et al., 1994). Therefore, one important contribution of GATA2 in this system may be to support hematopoietic cell survival and proliferation after commitment.

During development, hematopoietic cells descend from lateral plate mesoderm, which is marked by the expression of Flk1 in the mouse embryo (Sakurai et al., 2006a). Therefore, we first hypothesized that mesoderm cells were the source of hematopoiesis in our culture system. Following a similar reasoning, Nishikawa and colleagues grew ES cells on collagen IV coated plates in serum containing medium and separated $Flk1^{+}$ mesoderm cells to induce blood formation from ES cells (Nishikawa et al., 1998). However, the frequency of cells expressing Flk1 following co-expression of SCL, LMO2 and GATA2 is not sufficient to explain the boost in hematopoietic cell numbers. Furthermore, the expression of the mesodermal marker Brachyury, which is significantly lower than its expression in EBs, is further downregulated in response to transcription factor cocktail expression. From these, I concluded that the hematopoietic cells produced in this system did not go through a mesoderm intermediate.

An alternative explanation is that the transcription factor cocktail directly programs cells to the hematopoietic fate. A similar phenomenon was recently observed in the *Xenopus* embryos, where Chen and colleagues showed that expression of Runx1 can induce ectopic myelopoiesis without increasing the expression of the mesodermal markers (Chen et al., 2009).

An analysis of the expression of the germ layer markers showed severe reduction in all three germ layers, suggesting conversion of all cell types. This model is supported by the data obtained from the time course analysis of hematopoietic induction potential (Fig 4a). Even though the cell types in culture change over time, the level of the hematopoietic induction stays roughly the same from day 3 to day 8, suggesting all early cell types are affected similarly.

In other published examples, the complete programming of the lineage requires the expression of factors for an extended period. In our case, 24 hour expression of a cocktail of SCL complex members followed by 24 hours of uninduced culture was sufficient for strong, although not maximal, hematopoietic induction. It could be argued that the expression of three transcription factors, SCL, LMO2 and GATA2, could induce the expression of the hematopoietic target genes, like globins or CD41, without actually changing the fate of the cell. However, the data presented here shows that these markers were not induced directly by the complex, but rather were turned on as the cells matured in the second 24 hours of the culture in the absence of factor expression.

After the removal of the factors, most cells did not lose hematopoietic marker expression. Expression of the epiblast marker Fgf5 and the mesendodermal markers Brachyury and Goosecoid bounced back to the levels of the uninduced cells after three

days of culture without induction. On the other hand, the expression of ectodermal markers Pax6, Sox1 and Otx2 did not bounce back, suggesting a complete loss of the ectodermal fate. I hypothesize that the cells that were not completely programmed during the 24 hour expression period, reverted back to a developmentally nearby lineage, namely the mesendoderm.

The efficiency I observed might be the result of the plastic nature of early embryonic cells. It is not yet known whether the expression of these three factors can reprogram a fully differentiated somatic cell type, e.g. a fibroblast, but based on the loss of efficiency with later term inductions in monolayer cultures, it is likely that reprogramming a somatic cell type to blood will not be as straightforward. Such a conversion would likely require the removal of the epigenetic marks and thus either require more time or additional factors.

An unexpected result was the lack of response from the ES cells in the first two days of culture. During these days the self-renewal program of the ES cells is still active and I hypothesize that the differentiation signals are ignored as a result of this.

Having a simple system for the production of required cell types is crucial for therapeutic applications. The advantage of the monolayer differentiation over the EBs is scalability and ease of use in a clinical setting. Additionally, monolayer differentiation is expected to be less variable and to allow more precise control, compared to a system where complex intracellular interactions occur at a significant level. To our knowledge, this is so far the only report of efficient hematopoiesis from ES cells in a serum free monolayer culture system.

Monolayer differentiation is expected to be scalable, because it removes the rate-limiting EB formation step. However, genetic engineering to enable inducible gene expression is not suited for differentiation of ES cells for therapeutic purposes. An ideal method would induce hematopoiesis without a need for sort separation of populations or genomic engineering for gene expression. During the course of these experiments, I observed that certain lots of bovine serum, contained factors sufficient to induce hematopoiesis in the cultures of wild type ES cells. This suggests that the induction by extracellular signaling factors is possible.

The cells used in this study were engineered to express transcription factors inducibly. Genetic modification is both a risk factor and a rate-limiting step for clinical application. Recent studies in the reprogramming field have shown that genomic integration can be avoided by transfection of plasmid verctors (Yu et al., 2009) or transduction of recombinant proteins (Kim et al., 2009). Furthermore, we have shown that unlike pluripotency factors, expression of the hematopoiesis-inducing factors does not have to be continuous. Therefore, a one-time transfection of the expression plasmids, RNA or recombinant proteins may be sufficient for the induction. Similar methods to produce and study other lineages can be adapted.

CHAPTER 6: CONCLUSIONS AND FUTURE STUDIES

In this project I set out to answer two questions about the hematopoietic master regulator SCL: ***What effect does SCL have on cells in different stages of hematopoietic development?*** (Chapter 3) and ***Through what molecular mechanisms does SCL act in early development?*** (Chapter 4)

Work directed to answer the first question has been conducted in different non-mammalian models (Gering et al., 1998; Gering et al., 2003; Mead et al., 1998). A common finding of all the studies has been an increase in the hematopoiesis in response to the SCL over-expression. It has been suggested that SCL programs the mesoderm to a hematopoietic fate. However, none of these models are as flexible, practical, or easy to interpret as mouse ES cell differentiation. A combination of the tet-inducible gene expression and the ES cell differentiation allowed me to perform a detailed time course analysis in a mammalian model system.

I have found that ectopic SCL expression leads to an increase in the hematopoietic compartment and a decrease in other mesodermal lineages. I have shown this effect to be temporally limited to the mesoderm patterning stage of EB differentiation. Furthermore, I proved that this function is cell autonomous using chimeric EBs made up of iSCL and wild-type cells. In other words, SCL does not pattern the mesoderm by inducing the expression of a secreted morphogen, like BMP4 or Wnts. I also looked for signaling systems upstream of SCL and determined that SCL acts downstream of BMP4 by using the BMP inhibitor Noggin.

The molecular interactions required for SCL function have mostly been studied in hematopoietic cell lines. In order to answer the second question, I used the model established in the first part of the study.

I have shown that SCL-LMO2 interaction is necessary for the patterning of the mesoderm by SCL. Ectopic expression of LMO2 during mesoderm patterning stage increased hematopoietic output like SCL and a mutant of SCL that binds LMO2 weakly, did not affect hematopoiesis.

IP experiments were performed in cells where SCL was ectopically expressed in order to gather enough material for silver staining and mass spectrometry. This might have resulted in the formation of biologically irrelevant complexes. Therefore, confirmation of the candidate genes with IPs in wild-type ES cells is warranted. Knockout or knockdown phenotypes of candidate SCL interacting proteins during mesoderm patterning should also be performed to determine relevance.

We and others have shown that SCL-LMO2 interaction is required for mesoderm patterning (Gering et al., 2003), but it is still not clear why this interaction is necessary. Comparing proteins interacting with SCL in the presence or absence of LMO2 would reveal information on the exact role of LMO2 in the complex. This experiment could be performed through the immunoprecipitation of LMO2-binding deficient SCLF238G. However, SCLF238G still has some LMO2 binding activity (Schlaeger et al., 2004) and thus decreased binding of proteins should be expected, rather than a complete absence. Immunoprecipitation of SCL in the LMO2-/- cells or wild-type cells after LMO2 knockdown would also be worthwile. However, loss of LMO2 might result in the loss of

the relevant cell population, lateral plate mesoderm, and thus the findings might be biologically irrelevant.

The microarray analysis indicated distal primitive streak genes FoxA2 and BMP inhibitor Chordin and two members of the Id family, Id1 and Id3 as direct and novel targets of SCL during early differentiation.

Downregulation of distal primitive streak genes FoxA2 and Chordin by SCL offers a possible model for the patterning of mesoderm towards hematopoiesis, a proximal fate. It is also possible that SCL is necessary to stabilize the proximal fate by repressing genes associated with the distal fate. We do not yet know whether this downregulation is biologically relevant. Early streak embryos should be co-stained for SCL and FoxA2 expression to show whether they are co-expressed in the same cell or exist in a salt and pepper distribution, like GATA6 and Nanog in the ICM (Chazaud et al., 2006). Expression of FoxA2 in SCL-/- embryos (or vice versa) should also be evaluated.

My findings here on the downstream targets and interacting partners of SCL during early hematopoiesis can be used as a starting point to reveal a better picture of the early hematopoiesis.

I used a short induction before microarray analysis to enhance the detection of direct transcriptional targets. Nevertheless, ChIP to show direct binding of SCL to the promoters of the target genes is necessary to conclude direct regulation. SCL binding by

itself to target sites may not be sufficient to change the gene expression. For example, in erythrocyte progenitors, SCL complex was shown to bind the DNA without affecting the gene expression and only to activate it later in development. Using high-throughput methods in tandem with ChIP (ChIP-chip or ChIP-seq) may reveal additional SCL binding sites and additional targets might be revealed using this technique.

Another question remaining regarding the mesoderm patterning function of SCL is whether all the target genes require the binding of the same SCL complex. Preliminary data in chapter 4.d suggests that LMO2 binding is necessary for the repression of the targets I have identified here. ChIP experiments with LMO2 or GATA2 and comparison of binding sites to SCL ChIP results may identify sites bound by different types of SCL containing complexes.

In the final part of the study, I used the cues from developmental biology to ***develop a protocol to induce hematopoiesis efficiently in monolayer cultures*** (***Chapter 5***).

Directed differentiation of the pluripotent cells to specific lineages has gained importance in recent years. The generation of pluripotent cells from somatic cell types has opened up the possibility to create patient-specific cell lines and differentiate them back to a needed cell type for therapy. ES cells spontaneously differentiate *in vitro* to form a mixture of cells that normally descend from the three germ layers. Most ES cell differentiation protocols aim to mimic natural development to channel differentiation towards a specific cell type. However, due to the complexity of the process, these protocols usually require labor intensive steps, like embryoid body (EB) formation or

FACS separation, that are difficult to scale up, and reagents that are difficult to adapt for clinical use, like bovine serum. A major goal of regenerative medicine is the development of simple scalable methods for efficient differentiation of ES cells into specific cell types.

Somatic cells express specific gene sets that are necessary for their function, while the expression of most other genes is silenced. As cell fates become more specialized, more permanent epigenetic marks of chromatin, e.g. histone deacetylation, phosphorylation and methylation, are used to silence the unused portion of the genome. Thus it is thought that the transcriptome and hence the somatic cell type cannot be switched to another cell type easily. In recent times, cascades of transcription factor regulation governing the establishment of lineage-specific transcriptomes during development have been identified. A number of successful attempts based on this knowledge, like the expression of c/EBPα and c/EBPβ to reprogram B cells to macrophages (Xie et al., 2004); expression of MyoD in NIH3T3 cells to form myoblasts (Russo et al., 1998) and recently conversion of fibroblasts to ES-like pluripotent cells (Takahashi and Yamanaka, 2006), have shown that ectopic expression of certain transcription factors can change cell fate.

First, I showed that SCL can induce hematopoiesis in monolayer cultures, a function normally carried out by signals emanating from the visceral endoderm (Hochman et al., 2006). However, the level of induction was very low. Co-expression of LMO2 and GATA2 with SCL improved efficiency remarkably, and resulted in the routing of progenitors of other germ layers into the hematopoietic lineage with high efficiency.

Blood formation without genetic manipulation of the cells would be ideal for clinical purposes. My initial work with monolayer cultures was done with serum containing medium and in these experiments I have observed hematopoietic activity from wild-type ES cells. This suggests that soluble factors present in serum might also be used to induce hematopoiesis, if they can be identified.

BMP4 has been shown to induce SCL (Mead et al., 1998), LMO2 (Mead et al., 2001) and GATA2 (Maeno et al., 1996) expression. Therefore, theoretically, addition of BMP4 to monolayer culture system should mimic the expression of the transcription factor cocktail. Indeed, BMP4 has been shown to induce blood formation in the EBs grown under serum free conditions. However, the EBs and the animal explants might be secreting secondary signals in response to BMP4, which will disperse into the medium and thus may not be as effective in the monolayer culture system as they are in the EBs or *in vivo*. Comparisons between cultures either treated with BMP4 or induced to express the transcription factor cocktail by high-throughput systems, like microarray, will provide clues to find out the soluble or cell-autonomous factors responsible for the induction of blood fate in this culture system.

Hematopoietic cells produced from the mouse embryonic stem cells do not repopulate the hematopoietic system of the adult animals. Expression of some transcription factors like HoxB4 has been shown to allow cells to repopulate adults (Kyba et al., 2002). I have not tested the repopulation ability of SCL-LMO2-GATA2 induced hematopoietic cells, but it is highly likely that additional factors will be required to enable these cells to act as definitive HSCs. In colony assays the monolayer progenitors form colonies similar to those obtained from EBs cultured in serum. If the two

populations are proved to be identical after more rigorous testing, e.g. by comparison of their transcriptomes, the knowledge gained from the EB differentiation studies should be applicable to the production of transplantable cells from a monolayer culture.

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